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Aquatic Toxicity Tests to Characterize the Hazard of Volatile Organic Chemicals in Water A Toxicity Data Summary. Parts 1 and 2

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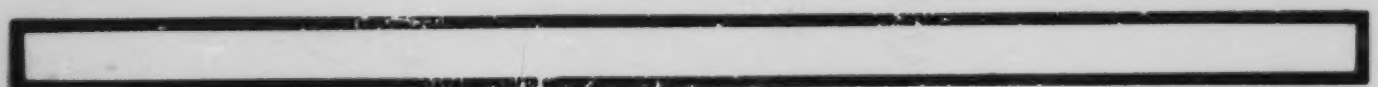

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AQUATIC TOXICITY TESTS TO CHARACTERIZE THE HAZARD OF VOLATILE ORGANIC CHEMICALS IN WATER: A TOXICITY DATA SUMMARY -- PARTS I AND II

by

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16. ABSTRACT

This summary presents acute and chronic toxicity test data and bioconcentration factors compiled over a 2-year period on fish and invertebrates exposed to several representative chemicals from 5 chemical classes (chlorinated ethanes, chlorinated benzenes, chlorinated ethylenes, chlorinated dienes, and chlorinated propanes).

The fathead minnows and Daphnia were quite similar in their sensitivities (acute and chronic) to each chemical class, while the rainbow trout were considerably more sensitive to all classes during acute tests, except for the chlorinated diene exposures, where they were more resistant. The ranking of acute and chronic sensitivity was generally the same for each chemical within each class of chemicals for all three species tested.

Both the acute and chronic toxicity of all chemicals within a class increased as the number of chlorines in the chemical structure increased.

Bioconcentration factors for fathead minnows were determined for four of the chemical classes tested. Hexachlorobenzene was bioconcentrated the most (23,000x) while tetrachloroethane was bioconcentrated the least (8x). Again, as with the toxicity experiments, the greater the number of chlorines on the molecule the greater the bioconcentration within each class of chemicals.

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INTRODUCTION

The objectives of this study were to develop bioassay methods, and provide information on the relative toxicity and metabolic relationships between selected aquatic organisms and higher animals. Investigations were divided into three major areas. The first phase involved determining similarities and differences in metabolism of selected xenobiotics between aquatic and mammalian organisms. Results of this work have been reported under separate cover (Ahmad et al., 1981). The second phase of the study was to develop methods for testing volatile chemicals, and to evaluate the sensitivity and similarity among daphnids, embryo-larval fish and mammals. The third phase was directed toward evaluating the use of a fish carcinogenesis model, involving the fathead minnow, as a predictor of environmental carcinogenesis.

Chemicals selected for testing were chosen from four classes of compounds - halomethanes, chlorinated ethanes, chlorinated benzenes and chlorinated ethylenes. These classes were suggested by personnel at HERL-Cincinnati who planned to study many of these same chemicals in mammalian systems. These data should be of particular interest to these people.

This report represents an overview of research results obtained by a number of different investigators. All data has been, or is scheduled to be, published in peer-reviewed scientific journals. A listing of scientific reports expected as a direct, or indirect, result of monies allocated for this unit of study follows.

PUBLICATIONS EXPECTED AS A RESULT OF THESE STUDIES

Ahmad, N., Catherine Moriarity and James Huot. 1981. Microsomal metabolism and binding of carbon tetrachloride, chloroform, 1,1,2-trichloroethane, 1,1,2-trichloroethylene and monochlorobenzene by microsomal fractions of rainbow trout and water flea. (Manuscript in preparation).

Benoit, D. A., F. A. Puglisi, and D. L. Olson. 1981. A compact continuous-flow mini-diluter exposure system for testing early life stages of fish and invertebrates in single chemicals and complex effluents. Water Res. (In press).

Benoit, D., F. Puglisi, and D. Olson. 1981. A fathead minnow early life stage toxicity test method evaluation and exposure to four organic chemicals. J. Environ. Pollut. (In press).

Benoit, D., R. Syrett, and F. Freeman. 1981. Design manual for construction of a continuous flow mini-diluter exposure system. (Submitted to USEPA for approval, April 24, 1981).

Carlson, A., and P. Kosian. 1981. Toxicity and bioconcentration of several chlorinated benzenes in fathead minnows. (Manuscript in preparation).

Carlson, A. 1981. Effects of low dissolved oxygen concentrations on the toxicity and bioconcentration of 1,2,4-trichlorobenzene in early life fathead minnows. (Manuscript in preparation).

DeFoe, D. 1981. Effects of four chlorinated ethanes and one chlorinated ethylene on survival and growth of fathead minnows. (Manuscript in preparation).

Richter, J. E., S. F. Peterson, and C. F. Kleiner. 1981. Acute and chronic toxicity of some chlorinated benzenes, ethanes, and tetrachloroethylene to Daphnia magna. (Manuscript in preparation).

Shubat, P., S. Poirer, M. Knuth, D. Hammermeister, A. Lima, L. Brooke, D. Call, and T. Felhaber. 1981. Acute toxicities of nine chlorinated organic compounds to selected freshwater organisms. (Manuscript in preparation).

Shubat, P., S. Poirer, M. Knuth, and L. Brooke. 1981. Acute toxicity of tetrachloroethylene and tetrachloroethylene with dimethylformamide to rainbow trout. Bull. Environ. Contam. Toxicol. (In press).

Veith, G., D. Call, and L. Brooke. 1981. Structure-activity relationship for estimating bioconcentration factors and acute toxicity with fish. (Manuscript in preparation).

Walbridge, C., J. Fiandt, G. Phipps, and G. Holcombe. 1981. Acute toxicity of ten chlorinated aliphatic hydrocarbons to the fathead minnow. (Manuscript in preparation).

METHODS

Acute Toxicity with Fish

Exposure System--Proportional diluters (Mount and Brungs, 1967) were used to carry out these tests. The dilution factors were 0.6 (that is each concentration, except the control, was 0.6 times the next higher concentration). With the five test concentrations used this covered a range of 2 orders of magnitude. All the chambers were duplicated. Flows were 3.2 to 10 tank-volumes per day. In general, all methods followed closely those of the committee for toxicity tests with aquatic organisms (1975).

Physical and Chemical Conditions--The dilution water was unfiltered Lake Superior water. The water temperature in the test tanks was held at a mean of 25 $\pm$ 0.7°C.

At least once during each 8-day test dissolved oxygen (DO), percent saturation, pH, hardness (as $CaCO_3$), and alkalinity were determined. The means, standard deviations and ranges for these variables, were: DO, 8.00, 0.37, 7.6-9.2 mg/l; pH, 7.37, 0.17, 6.7-7.6; total hardness, 45.08, 0.37, 45.0-45.5 mg/l $CaCO_3$; total alkalinity, 41.75, 1.56, 35.6-43.4 mg/l as $CaCO_3$.

Biological Methods--Fathead minnows (Pimephales promelas) were the experimental animals. These were laboratory-reared to an age of 30-35 days for use in these experiments. The rearing was done in a system similar to the exposure system. The rearing water was the same as the diluent water, with the temperature held at 25 $\pm$ 2 C. Fish in the rearing tanks were fed live brine shrimp nauplii in excess until 12 to 24 h before testing. The fish were not fed during the exposure period.

When starting a test 10-50 fish were randomly assigned to each of the 12 exposure tanks. Dead fish were counted and removed at least twice during the first day, and twice daily after that. The apparatus used was described in Phipps et al. (1981).

Methods not discussed here followed those specified by the U.S. Environmental Protection Agency (1975).

Chemical Methods--The chemical analyses for these compounds were performed by gas chromatography; 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, tetrachloroethylene, pentachloroethane, hexachloroethane, and hexachlorobutadiene were all run on a Hewlett-Packard 5730A automatic gas chromatograph equipped with a Model 3552A data system and a ^{63}Ni electron capture detector. The column was packed with 100/120 mesh Supelcoport® coated with 1.5% SP2250/1.95% SP-2401. The carrier gas was 5% methane in argon and the column temperature was adjusted between 40 and 80°C depending on the compound. Retention times varied between 1.50 and 5.00 minutes. The 1,2-dichloroethane, 1,2-dichloropropane, 1,3-dichloropropane, and 1,1,2-trichloroethylene were run on a Tracor MT-220 manual gas chromatograph with a ^{63}Ni electron capture detector. The column was packed with 80/100 mesh Gas-Chrom Q® coated with 4% SE-30/6% OV-210. The carrier gas, column temperatures, and retention times were the same as above. Gas chromatographic analyses on the benzene compounds were performed on a model 5730A Hewlett-Packard gas chromatograph equipped with an auto sampler, a ^{63}Ni electron capture detector, and a Hewlett-Packard Model 3354B laboratory automation data system. The column was 2.3 mm (I.D.) x 2 m packed with 1.5/1.95 percent SP-2250/SP-2401 coated Supelcoport (100-120 mesh). The carrier gas was 5 percent methane in argon. The injector and detector

temperatures were 250 and 300 C, respectively, and the oven temperature for each chemical is presented in Table 1.

Water samples were added directly to 100 ml volumetric flasks to which 50 ml of hexane had already been added. The samples were then stirred vigorously on magnetic stirring devices for 1.5 h and allowed to separate for 1 h. The samples were then diluted with hexane if necessary and analyzed as outlined above by comparison to known hexane standards. Known amounts of the chemicals were added to water samples to determine the efficiency of this extraction procedure and the recoveries exceeded 95%. Final results were not corrected for recovery.

Statistical--The LC50 concentrations were calculated by using the Trimmed Spearman-Karber method for estimating median lethal concentrations (Hamilton et al., 1977).

Acute Toxicity to Rainbow Trout

Exposure System--Lake Superior water was used for all tests. It was modified only by heating or cooling portions and mixing them together in the proportions necessary to yield the desired test temperatures. Temperature was controlled within $\pm$ 1.0°C of nominal test temperatures.

Fish were exposed in a flow-through diluter with room air temperature maintained at 12°C, and a controlled photoperiod of 16 hr light (28-29 ft.c.). Test chamber water exchange rates ranged from 3.2 to 9.3 times per 24 hr. Test chamber dimensions (I.D.) were 21.0 x 35.0 x 24.5 cm, and contained water at a depth of 9.0 cm (pentachlorobenzene and hexachlorobenzene were exceptions). Ten fish were exposed per chamber resulting in chamber loadings ranging from 1.3 to 4.1 g/L. Five exposure concentrations and a control were used for all tests except hexachlorobenzene.

TABLE 1. SUMMARY OF ANALYTICAL CONDITIONS AND RECOVERIES.

Chemical	Sample Vol (ml)	Extract Vol (ml)	GLC Temp. (°C)	Spiked Recoveries (a) Water (%)	Tissue (%)
Hexachloroethane	200	150	80	109	89
Pentachloroethane	200	150	60	N.D.	86
1,1,2,2-Tetrachloroethane	200	150	60	96	82
Tetrachloroethylene	200	150	40	N.D.	74
1,1,2-Trichloroethane	200	150	40	96	N.D.
Hexachloro-1,3-butadiene	200	150	100	95	96
Hexachlorobenzene	100	50	160	98	91
1,2,3,4-Tetrachlorobenzene	100	50	80	100	94
1,2,4-Trichlorobenzene	100	50	130	99	92
1,3-Dichlorobenzene	100	50	80	102	95
1,3-Dichlorobenzene	100	50	80	107	99

Rainbow trout tests with pentachlorobenzene and hexachlorobenzene were run in a diluter at 12 C (nominal) water temperatures. Two toxicant concentrations (saturation and 10 times saturation, nominally) and a control, all in duplicate, were tested in 30.0 x 60.0 x 15.0 cm glass chambers containing 27.0 L of water. The water metering cells delivered 1.0 L of water to each test chamber every 8.2 minutes and the toxicant was delivered simultaneously by metering pumps into mixing chambers before entering the exposure chamber. Stock solutions of dimethylformamide (DMF) containing the appropriate amounts of hexachlorobenzene were prepared for each pump enabling each exposure chamber to receive the same amount of DMF and different amounts of hexachlorobenzene. Both hexachlorobenzene and DMF concentrations were measured in the water. Ten fish were tested in each chamber for 96 hrs with a photoperiod of 16 hr light. For each test, all exposures and controls were duplicated. Mortalities were observed and recorded at 1, 2, 4, 8, 12 and 24 hr and daily thereafter.

Physical Chemical Conditions--Total hardness, acidity, total alkalinity (all as mg/L as $CaCO_3$), pH, and dissolved oxygen (mg/L) were measured several times at 3 or more exposure concentrations in test chambers during each test. Exposure chamber water temperatures were measured daily.

The ranges for all water chemistries were: total hardness - 50.6 to 56.8 mg/L as $CaCO_3$; total alkalinity - 44.6 to 53.1 mg/L as $CaCO_3$; acidity - 1.97 to 4.1 mg/L as $CaCO_3$; pH - 6.8 to 7.5; dissolved oxygen - 8.0 to 9.6 mg/L; and temperature - 11.6 to 12.7°C.

Chemical Methods--Chamber water concentrations of the toxicants were measured in all chambers twice during each test (i.e., at the start and at 96 hrs). On the other days of exposure one chamber of each replicate was measured for toxicant concentrations. 1,2,4-trichlorobenzene was extracted

into petroleum ether and 1,2-dichlorobenzene, and 1,4-dichlorobenzene were extracted into hexane and analyzed by GLC. Water samples of 2-50 ml are placed into 100 mL volumetric flasks with 50 mL of organic solvent. The total volume is brought up to 100 mL with distilled water, stirred for 20 min on a magnetic stirrer, and diluted as appropriate for GLC analysis.

GLC analyses were performed on a Tracor 550 gas chromatograph with a ^{63}Ni detector and column packing of 3% OV-101 on 100/120 mesh Gas Chrom Q and N_2 carrier gas flow rate of 50 mL/min. At a column temperature of 120 C, 1,2,4-trichlorobenzene had a retention time of 0.61 min.

Recovery of 1,2,4-trichlorobenzene from Lake Superior water spiked over a range of concentrations from 0.1 to 10 mg/L was 96.8 $\pm$ 2.0% for 12 analyses. Recovery of 1,2-dichlorobenzene from Lake Superior water spiked over a concentration range of 8.4 µg/L to 8.4 mg/L was 103.7 $\pm$ 2.6% for 15 determinations, and recovery of 1,4-dichlorobenzene spiked into Lake Superior water over a concentration range between 0.2 to 20 mg/L was 100.1 $\pm$ 3.0% for 19 determinations.

Hexachloro- and pentachlorobenzene concentrations in water were determined by extraction into hexane and analysis by GLC. Water samples of 1-5 mL are placed into 18 mL glass-stoppered test tubes, 3 drops of saturated NaCl solution was added, followed by the addition of 5.0 mL hexane. The tubes were shaken for 3 minutes, and diluted as necessary for GLC analysis.

GLC analyses were performed on a Tracor MT 160 gas chromatograph equipped with a ^{63}Ni electron - capture detector and a column packing of 3% OV-101 on 100/120 mesh Gas Chrom Q. At a column temperature of 205 C and a N_2 carrier gas flow rate of 50 mL/min, retention times were 2.41 and 1.18 min for hexachloro- and pentachlorobenzene, respectively.

Recovery of hexachlorobenzene from Lake Superior water spiked with test compound over a concentration range from 1.0 µg/L to 1.0 mg/L was 96.6 ± 3.1% for 23 determinations. Recovery of pentachlorobenzene spiked into Lake Superior water over a concentration range from 10.0 µg/L to 10.0 mg/L was 94.3 ± 4.0% for 13 determinations.

For the hexachlorobenzene, pentachlorobenzene, and tetrachloroethylene acute tests in which DMF was the stock solvent, levels of DMF in exposure chambers were determined. DMF was analyzed on a UV-visible double-beam spectrophotometer at a wavelength of 200 nm.

Water samples containing hexchloroethane and tetrachloroethylene were extracted by adding 5.0 to 50.0 mL of sample to a 100 mL volumetric flask containing 50.0 mL of hexane. Samples less than 50.0 mL were diluted to volume with distilled water. The samples were stirred vigorously for 20 minutes on a magnetic stirrer. Samples were allowed to stand 15 minutes, then diluted as necessary for GLC analysis.

GLC analysis was performed on a Tracor 550 instrument equipped with a ^{63}Ni electron-capture detector. The 180 cm x 4 mm column was packed with 3% OV-101 on 100/120 mesh Chromosorb® W. The carrier gas was argon-methane (95:5) at a flow rate of 50 mL/min. All peak area calculations were performed by a Hewlett-Packard Laboratory Automation Data System. Detector and inlet temperatures were 300 C and 225 C, respectively. Column temperatures for hexachloroethane and tetrachloroethylene were 130 and 65 C, respectively.

The retention time of hexachloroethane was 1.30 minutes and the sensitivity was about 1 pg at an attenuation of 32X. The retention time of tetrachloroethylene was 1.60 minutes and the sensitivity was about 1 pg at an attenuation of 64X.

Recovery of hexachloroethane from Lake Superior water spiked with the test compound over a concentration range of 0.20 to 2.0 µg/mL was 96.7 + 2.9% for 16 determinations.

Recovery of tetrachloroethylene from Lake Superior water spiked with the test compound over a concentration range of 0.086 to 43.2 µg/mL was 89.9 ± 6.2% for 23 determinations.

Water samples containing 1,3-hexachlorobutadiene were extracted by adding 75.0 ml of samples to a 100 mL volumetric flask containing 25.0 mL of isooctane, and stirring vigorously for 20 minutes on a magnetic stirrer. The samples were allowed to stand for 15 minutes, then diluted as necessary for GLC analysis.

GLC analysis was performed on a Tracor 550 instrument equipped with a ^{63}Ni electron-capture detector. The 183 cm x 6 mm column was packed with 3% OV-101 on 80/100 mesh Chromosorb® W. The column oven was operated isothermally at 220 C. Detector and inlet temperatures were 300 C and 215 C, respectively. The carrier gas was argon-methane (95:5) at a flow rate of 50 ml/min. A Hewlett Packard automatic sampler was modified to fit the Tracor GLC and all calculations were performed by a Hewlett Packard Laboratory Automation Data system. The senstivity of 1,3-hexachlorobutadiene was about 1 pg at an electrometer attenuation of 16X. The retention time with the above conditions was 2.35 minutes.

Statistical--The LC50 concentrations were calculated by using the trimmed Spearman-Karber method for estimating median lethal concentrations (Hamilton et al., 1977).

Acute Toxicity with Daphnia

Exposure System--Adult daphnids (Daphnia magna) were originally obtained from the laboratory stock reared at the U.S. Environmental Protection

Agency, Duluth, MN. All culturing and testing were done using Lake Superior water which was filtered (<5μm), heated to 20°C, and aerated with filtered air. Means and ranges for total hardness and total alkalinity of test waters were 44.7 (43.5-47.5) and 41.5 (37.0-45.5) mg/L as $CaCo_3$, respectively. Chemical measurements were made in accordance with procedures in American Public Health Association (1975). Additional chemical characteristics of Lake Superior water are summarized in Biesinger and Christensen (1972). Culturing and testing were done in an enclosed constant temperature water bath (20 + 1 C). A combination of Gro-Lux and Duro-Test (Optima FS) fluorescent bulbs provided 344 lumens at the air water interface and were on a 16L:8D photoperiod coupled with a 15 minute transition period between light and dark phases. Brood cultures of 25 animals in 1 L beakers were maintained by renewing food (30 mg/L) and water three times each week. For acute and chronic testing, first instar daphnids (<24 hours old) were collected from brood animals of approximately 3 weeks in age.

Chemical stock solutions were prepared by saturating lake water with the test chemical on a magnetic stirrer plate.

Acute bioassays were conducted according to the ASTM "Standard Practice of Conducting Basic Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians" (ASTM 1979). Test containers were 200 mL erlenmeyer flasks filled to 200 or 160 mL for unfed and fed tests, respectively. The flasks were tightly stoppered with foil wrapped neoprene stoppers. Food concentration was 20 mg/L. Acute toxicity endpoints were the 48 hr median effective concentration (48 hr EC50) determined by complete immobilization, and the 48 hr lethal concentration (48 hr LC50) based on death, determined by cessation of heart beat and gut movement. Both endpoints were determined using a 30x dissection scope.

Physical-Chemical Conditions--Oxygen was measured with either a Beckman Model 0260 Oxygen Analyzer or by Winkler titration. pH measurements were made with a Corning Model 12 pH meter. These measurements were generally made at low, medium, and high toxicant concentration of both new and old samples. Total alkalinity and total hardness measurements were made according to APHA (1975). All values of these measurements fell within the ranges given for rainbow trout on page 9.

Chemical Methods--All chemicals used in preparing standards were taken from the same stock bottle as those used for the exposure test system. The chemicals were purchased from the Aldrich Chemical Company and ranged in purity from 95 to 99 percent. The solvents, hexane, iso-octane, and acetone were purchased from Burdick and Jackson Laboratories, Inc. and were glass distilled gas-chromatography grade. Standards and spike solutions were weighed with a Sartorius analytical balance and prepared in 100 mL volumetric flasks. Because of the volatility of the chemicals being tested, both the standards and the spike solutions were refrigerated while not in use and renewed after one month.

Water samples were taken three times a week. The samples included both the initial and final concentrations of the exposure water in the renewal static test system. Seventy-five mL of sample from selected test bottles were transferred with the aid of a funnel to 100 mL volumetric flasks containing 25 mL of hexane. A Teflon-coated magnetic stirring bar was placed in each flask and stirred rigorously for one hour with at least half of the solvent in suspension. When necessary the samples were stored in a refrigerator for no longer than three days.

The effectiveness of the extraction method was examined by determining the percent recovery of a known amount of chemical in water. The recoveries

for several chemicals ranged from 91 to 103 percent. Duplicate samples of the same test bottle and concentration were also used to determine the accuracy of the overall analytical method. Accuracy of the analytical method was within 92 percent.

A Hewlett-Packard 5710A gas chromatograph equipped with an autosampler, a Hewlett-Packard 3354C data system, and a ^{63}Ni pulsed electron capture detector was used for the analysis. The computer system was capable of automatically injecting the samples, integrating the detector response, calibrating standards, analyzing a set of samples, and storing the data. A 6 foot by 2 mm (ID) glass column packed with 80/100 mesh Gas Chrom Q® coated with 1.5% OV-17 plus 1.95% QF-1 was used with the following compounds and their respective isothermal oven temperatures: 1,1,2,2-tetrachloroethane (75 C), hexachlorobenzene (150 C), 1,2,4-trichlorobenzene (110 C), pentachloroethane (90 C), and hexachloroethane (100 C). A 6 foot by 2 mm (ID) glass column packed with 80/100 mesh Gas Chrom Q® coated with 4% SE 30/6% OV-210 was used for the additional compounds and their respective isothermal oven temperatures: 1,3-dichlorobenzene (110 C), 1,1,2-trichloroethane (50 C), 1,2-dichloroethane (50 C), and tetrachloroethylene (50 C). For all compounds the injection port temperature was 200°C and the detector temperature was 300 C. The carrier gas was 5% methane in argon with a flow rate of 41.7 mL/min.

Statistical--EC50 and LC50 values were derived using the measured mean effective toxicant concentrations (average initial and final test solution concentrations) and were calculated by probit, moving average, or binomial formulas depending on the characteristics of the data.

Development of Early Life Stage, Mini-Diluter Apparatus

In order to successfully and safely test volatile chemicals it was necessary to develop specialized exposure systems and methods for testing air and water leaving these systems. Interest in developing the early life stage (ELS) fish toxicity test led to the design of a compact continuous flow mini-diluter exposure system which accurately delivers as little as 3 liters of test water per hour to each of 5 concentrations plus a control. This system can be used to test the effects of either single chemicals or treated complex effluents on young fish in the laboratory or in the field. The small ELS test apparatus takes less space and requires smaller volumes of test water which is a critical factor when shipping effluents to the laboratory or conducting on-site toxicity tests. Smaller volumes of test water also reduces filtration costs when one is required to remove hazardous test chemicals before discharging waste water to the sewer.

The ELS test system has been tested and evaluated in the laboratory and on-site in a mobile trailer. This apparatus has been used to conduct fathead minnow (Pimephales promelas) ELS exposures to various toxicants including volatile organic compounds, metals, pesticides, and treated complex effluents from metal plating, oil refinery, and sewage treatment plants. The system also has been successfully used for testing macroinvertebrates.

Figure 1 shows a photograph of the compact stationary vented exposure system for conducting ELS tests. The vented plywood enclosure is sealed with fiberglass or epoxy paint on the inside and measures 76 cm wide x 120 cm long with a height of 112 cm over the exposure chambers and 159 cm over the dilter. Both apparatus and test fish can easily be observed through viewing windows located on the sides and top. One 5 cm hole located near the bottom of each side allows a continuous flow of air to be drawn through the

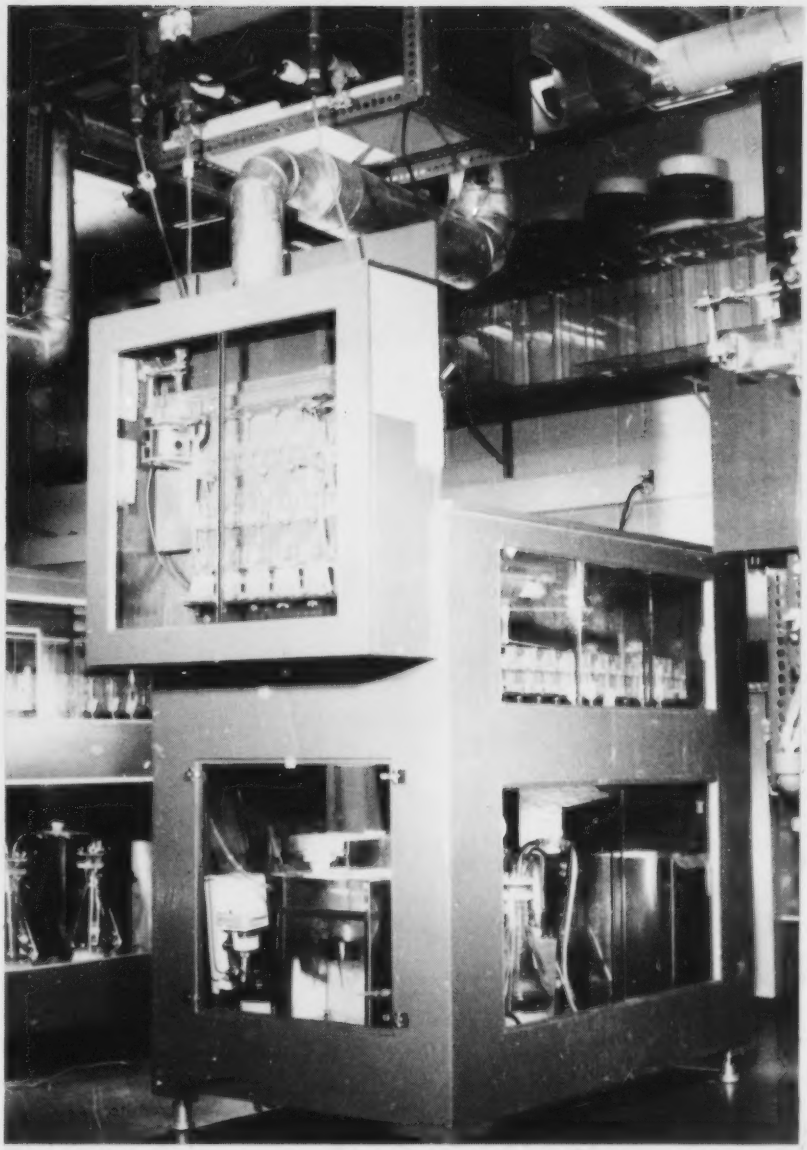

FIGURE 1: Mini-diluter exposure system for conducting early life stage toxicity tests.

enclosure and out a 10 cm exhaust vent located over the diluter. Exhaust air can be purified through a charcoal filter if necessary. Ample space is available in the bottom of the enclosure to install apparatus such as chemical saturators, stock bottles, metering pumps, or special filters used to remove the test chemical before discharging waste water to the sewer.

This system is ideally suited for use in testing hazardous volatile chemicals and was designed to protect the investigator from possible harmful exposures to toxic fumes. Negative pressure created on the inside of the enclosure enables one to safely service the system and take care of the test fish through small sliding glass doors. During tests conducted at our laboratory the enclosure was vented through the laboratory air exhaust system which drew an average of 0.7 cubic meters per min through the enclosure (approximately one air volume every 2 min). Air samples taken with one 30 x 30 cm sliding glass door open have shown measurable quantities of volatile test chemical inside the enclosure but no detectable concentrations were found outside.

Another feature of the enclosure is if the diluter leaks or overflows, the spilled test water can be diverted directly to the system's drain lines and will not flood the room. Due to the fiberglass or epoxy paint, the enclosure bottom is also water tight and can hold up to 100 liters if a leak should occur in some other part of the system. An alarm can be installed in the enclosure base to warn the investigator of major leaks. The accumulated test water in the base can then be drained off through a discharge valve and if necessary passed through a filter.

Design details of the mini-diluter exposure system has been written (Benoit, Syrett and Freeman, 1981) and submitted to U.S. EPA for approval as a design manual (for Appendix A). This manual compliments a paper,

undergoing peer-review by scientific journal editors, titled "A Compact Continuous Flow Mini-Diluter Exposure System for Testing Early Life Stages of Fish and Invertebrates in Single Chemicals and Complex Effluents" by Benoit, Mattson, and Olson (1981).

Chronic Toxicity with Fish

Exposure system--All tests were conducted with the above described mini-diluter exposure system installed in a vented enclosure. The small system incorporated four replicate glass exposure chambers (18.7 x 7 x 9.2 cm high) at each of five concentrations plus control. The mini-diluter delivered 15 mL of test water per min to each replicate 500 mL chamber. Test water delivery tubes were positioned by stratified random assignment. Replacement time for 90% of the test water was calculated to be approximately 75 min in exposure chambers (Sprague, 1969). Water depth in each chamber measured 4.5 cm. All test chambers were carefully siphoned daily with a large pipette and squeeze bulb, after larvae began feeding. Cleaning was done just before the last feeding of the day. Cool white fluorescent lamps were used as the main source of illumination and a constant daylight photoperiod of 16 hr was maintained. Light intensity at the water surface ranged from 30 to 60 lumens.

Physical-Chemical Conditions--Water obtained directly from Lake Superior was passed through a sand filter and ultraviolet sterilizer; and then heated to a test temperature of 25±1 C. Total hardness, alkalinity, acidity, and pH were determined on water from the control chambers once a week; dissolved oxygen was measured in each treatment twice a week. The means for total hardness, alkalinity, and acidity determinations were 45, 42, and 3 mg/L as $CaCO_3$, respectively. The mean dissolved oxygen was measured at 7 mg/L and

the mean pH equaled 7.4. Chemical measurements were made according to the American Public Health Association et al. (1975).

Chemical Methods--Saturated water solutions of hexachlorobutadiene, 1,2-dichloropropane, 1,3-dichloropropane, 1,2-dichloroethane, hexachloroethane, pentachloroethane, 1,1,2,2-tetrachloroethane, tetrachloroethylene, 1,1,2-trichloroethane, hexachloro-1,3-butadiene, hexachlorobenzene, 1,2,3,4-tetrachlorobenzene, 1,2,4-trichlorobenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene (98-99% purity)[1,2] were used as the toxicant source to avoid the use of solvents. Test chemical stock solutions during each test were continuously made up with a chemical saturator similar to one described by Gingerich et al. (1979). Stock solutions were delivered from the saturator to the diluter by an FMI[3] metering pump. Test concentrations were assigned to each exposure chamber by stratified random assignment.

All test water treatments were measured twice a week in alternate replicate exposure chambers. Chemical analyses of the test water samples containing hexachlorobutadiene, 1,2-dichloropropane, 1,3-dichloropropane, and 1,2-dichloroethane were done by solvent extraction followed by gas chromatography as described for acute tests with fish. Comparisons of chemical concentrations within each group of four replicates, sampled simultaneously, showed that concentrations were within 90% of each other (range, 85-98%). Samples from selected replicates were also split and

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[1] The U.S. Environmental Protection Agency neither recommends nor endorses any commercial product; trade names are used only for identification.

[2] Aldrich Chemical Co., Milwaukee, WI 53233

[3] Fluid Metering, Inc., Oyster Bay, NY 11771

analyzed separately to check the variability of the method used to measure test water concentrations. Results from eight split samples showed that reproducibility of the chemical analysis was within 98% (range 96-99%). Detection limits and percentage recovery of 8-11 spiked samples for hexachlorobutadiene, 1,2-dichloropropane, 1,3-dichloropropane, and 1,2-dichloroethane were 0.03 µg/L and 97% (range 82-109%), 0.09 mg/L and 99% (range 93-106%), 0.1 mg/L and 100% (range 97-107%), and 0.1 mg/L and 102% (range 97-105%), respectively. Because of the similarity of the other eleven chemicals tested the methods are described in general terms and specific conditions for each chemical are presented in Table 1. The individual measurements are presented in subsequent tables.

Water samples were siphoned directly from the tanks into volumetric flasks to which hexane had previously been added. After filling to the volumetric mark, a Teflon stirring bar was added and the sample was extracted by vortex mixing with a magnetic stirrer for 1.5 hours. The phases were allowed to separate for 0.5 hours, and an aliquot was removed, diluted if necessary, and transferred to a GLC sample injection vial for analysis. The calculation of water concentration was based on original volume of hexane pipetted into the volumetric flask before filling with water.

Gas chromatographic analyses were performed on a model 5730A Hewlett-Packard gas chromatograph equipped with an auto sampler, a ^{63}Ni electron capture detector, and a Hewlett-Packard Model 3354B labortory automation data system. The column was 2.3 mm (I.D.) x 2 m packed with 1.5/1.95 percent SP-2250/SP-2401 coated Supelcoport (100-120 mesh). The carrier gas was 5 percent methane in argon. The injector and detector temperatures were 250°C and 300°C, respectively, and the oven temperature for each chemical is presented in Table 1.

Statistical--Effect, no effect endpoints were determined as described in Benoit et al. (1981).

Bioconcentration--Bioconcentration factors were calculated for those chemicals listed in Table 1 on fathead minnows exposed for 28-days. Because of the large number of samples and the small size of the individual fish, surviving fish from each test concentration were composited into single samples for the determination of tissue residues. Whole fish samples were homogenized with 70 gms of anhydrous sodium sulfate previously cooled to about ~5°C. The homogenate was transferred to a 300 mL Shell column and extracted by eluting the column with 250 mL hexane collected in a 250 mL volumetric flask. An aliquot was diluted to an appropriate volume for analysis. Gas chromatographic analyses were performed on these samples as described earlier for water samples.

Chronic Toxicity with Daphnia

Exposure system--Chronic bioassays (28-day) were conducted according to the ASTM "Proposed Standard Practice for Conducting Static Renewal Life Cycle Toxicity Tests with the Daphnid, Daphnia magna" (ASTM, 1979), with minor modifications to control volatile chemical losses. Test containers were 200 mL Erlenmeyer flasks filled to 160 mL, with the exception of tetrachloroethylene which was filled to 175 mL. The flasks were tightly stoppered with foil wrapped neoprene stoppers. All of the flasks were held in a constant temperature bath under a specified photoperiod as descirbed earlier under acute toxicity with Daphnia.

Physical-chemical conditons--Identifical to those described for acute toxicity with Daphnia.

Biological methods--Each flask contained one daphnid. Food concentration was 20 mg/L. The tests with 1,1,2,2-tetrachloroethane,

1,3-dichlorobenzene, 1,2,4-trichlorobenzene had seven replicates at each of six test chemical concentrations, whereas 1,2-diochloroethane, 1,1,2-trichloroethane, and tetrachloroethylene had 10 replicates at each of six chemical concentrations. Young daphnids were filtered from each flask after the transfer of the adults and washed onto a watch glass to be counted alive with an Artek Counter•. If less than 20 animals are present they were counted visually. Chronic toxicity was determined by reproductive success and length of animals surviving the 28 day test. Counting the animals alive eliminated the additional steps of poisoning and stirring to redisperse them. This technique also allowed the determination of live from dead animals. Length was determined using a 30x dissection scope and measuring from the top of the head to the base of the spine with an ocular micrometer.

Statistical--Both reproductive success and length were treated statistically by analysis of variance and Dunnett's test. A NOEC (no observable effect concentration) was determined to be the highest concentration tested which was not significantly different from the control values at either $P \leq 0.05$ or $P \leq 0.01$. This concentration was the mean effective exposure based on measured new (at renewal) and old (before renewal) values through the entire 28 day test.

RESULTS AND DISCUSSION

Acute Toxicity Fish

Fathead minnows--The 96-hr LC50 values and 95% confidence intervals of these chlorinated aliphatic compounds are given in Table 2.

The most acutely toxic compounds tested were hexachlorobutadiene, 1,2,3,4-tetrachlorobenzene, hexachloroethane, and 1,2,4-trichlorobenzene with 96-hr LC50s of 0.10, 1.07, 1.53, and 2.76 mg/L, respectively. All other compounds in the group were considerably less toxic. Two of the compounds, hexachlorobenzene and pentachlorobenzene, were found to be acutely non-toxic near water saturation; therefore, no 96-hr LC50 could be determined (Table 2). Acute toxicity increased in direct relation to the number of chlorines on the molecule for the ethanes, benzenes, and ethylenes. The position of the chlorine on the molecule made a difference in acute toxicity with 1,3 and 1,4-dichlorobenzene, but seemed to have little effect on the 1,2 and 1,3-dichloropropanes.

Rainbow trout: 1,2-Dichlorobenzene--Rainbow trout from Lake Mills, Wisconsin, National Fish Hatchery (mean standard length, 5.6 cm; mean weight 2.7 g) were exposed to five concentrations (0.72, 1.26, 2.01, 3.07, and 3.81 mg/L) in duplicate, plus controls. Only one fish died beyond 48 hrs of exposure. The 96-hr LC50 value was 1.61 mg/L (Table 3). Fish that were unable to swim and laid motionless on the exposure chamber bottom were considered affected. The 96 hr EC50 value was 1.55 mg/L.

1,4-Dichlorobenzene--Rainbow trout fingerlings from Lake Mills, Wisconsin, National Fish Hatchery (mean length 52.7 $\pm$ 6.4 cm, mean weight 2.1 $\pm$ 1.0 g) were exposed to five concentrations of dichlorobenzene (1.74, 1.36, 0.83, 0.52, and 0.37 mg/L) in duplicate. The 96-hr LC50 was 1.12 mg/L (Table

TABLE 2. RESULTS OF FLOW-THROUGH ACUTE TOXICITY TESTS (MG/L) WITH FATHEAD MINNOWS EXPOSED TO 16 CHLORINATED ALIPHATIC COMPOUNDS.

Compound	24 h LC50 (mg/L)	48 h LC50 (mg/L)	72 h LC50 (mg/L)	96 h LC50 (mg/L)
Chlorinated Ethanes				
Hexachloroethane	1.80[a] (1.70-1.91)	1.55 (1.47-1.63)	1.55 (1.47-1.63)	1.51 (1.43-1.58)
Pentachloroethane	7.72 (7.45-7.99)	7.43 (7.16-7.71)	7.34 (7.07-7.63)	7.34 (7.07-7.63)
1,1',2,2'-tetrachloroethane	22.8 (21.9-23.8)	22.2 (21.2-23.1)	20.4 (20.0-20.8)	20.4 (20.0-20.9)
1,1',2-trichloroethane	81.6 (-)[b]	81.6 (-)	81.6 (-)	81.6 (-)
1,2-dichloroethane	141 (131-153)	118 (111-125)	116 (110-123)	116 (110-123)
Chlorinated Benzenes				
Hexachlorobenzene				-[c]
Pentachlorobenzene				-[c]
1,2,3,4-trichlorobenzene				1.07
1,2,4-trichlorobenzene				2.76 (2.62-2.91)
1,3-dichlorobenzene				7.79
1,4-dichlorobenzene				4.16
Chlorinated Ethylenes				
Tetrachloroethylene	17.9 (17.3-18.4)	15.9 (15.0-16.8)	14.9 (13.9-15.8)	13.4 (12.4-14.4)
1,1',2-trichloroethylene	58.8 (57.8-59.7)	57.9 (57.2-58.6)	55.4 (53.0-57.8)	45.0 (41.9-48.4)
Chlorinated Propanes				
1,3-dichloropropane	133 (126-139)	131 (124-137)	131 (124-137)	131 (124-137)
1,2-dichloropropane	194 (184-205)	154 (144-166)	141 (132-151)	140 (131-150)

TABLE 2. (Continued)

Compound	24 h LC50 (mg/L)	48 h LC50 (mg/L)	72 h LC50 (mg/L)	96 h LC50 (mg/L)
Chlorinated Butadienes				
Hexachlorobutadiene	-	0.23 (0.20-0.26)	0.13 (0.09-0.18)	0.10 (0.09-0.11)

[a] 95% confidence limits.

[b] Here it was not possible to calculate confidence limits. There were no partial kills. Mortality was either 0 or 100%.

[c] Not toxic at the highest concentrations that could be maintained in the chambers.

TABLE 3. MEAN AND 95% CONFIDENCE INTERVALS FOR THE 96 HR 50% EFFECT CONCENTRATIONS (EC50) AND 50% LETHAL CONCENTRATIONS (LC50) FOR RAINBOW TROUT (SALMO GAIRDNERI) EXPOSED IN LAKE SUPERIOR WATER TO VARIOUS ORGANIC COMPOUNDS.

Compound	96-hr EC50[a] (mg/l) Mean	95% Confidence Interval	96 hr-LC50 (mg/l) Mean	95% Confidence Interval
Chlorinated Ethanes				
Hexachloroethane	0.84	0.75-0.94	0.84	0.75-0.94
Chlorinated Benzenes				
Hexachlorobenzene	[b]		[b]	
Pentachlorobenzene/DMF[c]	0.10[d]	0.09-0.12	0.27[d]	0.20-0.37
1,2,4-Trichlorobenzene	1.27	1.11-1.46	1.52	1.34-1.72
1,4-Dichlorobenzene	1.10	1.05-1.16	1.12	1.05-1.20
1,2-Dichlorobenzene	1.55	1.44-1.65	1.61	1.48-1.77
Chlorinated Ethylenes				
Tetrachloroethylene	4.86	(?)	4.99	4.73-5.27
Tetrachloroethylene/DMF[c]	5.76	4.71-7.05	5.84	5.05-7.67
Chlorinated Butadienes				
Hexachlorobutadiene	0.14	0.13-0.15	0.32	0.13-0.15

[a] Abnormal swimming behavior, usually loss of equilibrium.

[b] No effects on lethality observed at water saturation.

[c] Compound was administered as a mixture with dimethylforamide to facilitate solubility.

[d] 144-hr LC50 due to insufficient death at 96 hrs to comute a LC50.

3). Loss of equilibrium occurred as much as 12 hrs before death and was recorded as an effect. The 96-hr EC50 concentration was 1.10 mg/L.

1,2,4-Trichlorobenzene--Rainbow trout fingerlings from Lake Mills, Wisconsin, National Fish Hatchery (mean length 47.0 + 4.0 cm, mean weight 1.55 ± 0.42 g) were exposed to five concentrations of trichlorobenzene (2.82, 1.68, 1.10, 0.58, and 0.43 mg/L) in duplicate. The 96-hr LC50 concentration was 1.52 mg/L (Table 3). Loss of equilibrium in the fish occurred as much as 48 hrs before death and was recorded as an effect. The 96-hr EC50 concentration was 1.27 mg/L.

Pentachlorobenzene/DMF--Rainbow trout from Fattig Hatchery, Brady, Nebraska (mean standard length, 6.9 cm; mean weight, 5.2 g) were exposed to five concentrations of pentachlorobenzene (59, 120, 277, 435, and 714 µg/L) in duplicate, plus controls. Saturation of Lake Superior water with pentachlorobenzene at 16.3°C was 325 µg/L. DMF was used as a solvent in all concentrations. DMF concentrations were nominally equal and averaged 395 mg/L between exposure chambers. The first death occurred after 48 hrs of exposure but there were insufficient deaths to calculate a 96-hr LC50 concentration. The test was run for 144 hrs and the LC50 concentration for this time was 0.27 mg/L (Table 3). Fish that had lost equilibrium or were motionless on the chamber bottom were considered affected. The 144-hr EC50 concentration was 0.10 mg/L respectively.

Hexachlorobenzene/DMF--Rainbow trout from Lake Mills, Wisconsin National Fish Hatchery (mean standard length, 33 + 3 cm; mean weight 0.46 ± 0.11 g) were exposed to two concentrations (3.8 and 80.9 µg/L) of hexachlorobenzene in duplicate plus controls. All exposure chambers including controls contained similar concentrations of DMF (932 ± 12.9 mg/L). Fish did not die or show signs of distress in any test concentrations in a 96-hr exposure.

Hexachloroethane--Rainbow trout from Fattig Hatchery, Brady, Nebraska (mean standard length, 66.4 ± 9.9 cm; mean weight, 4.3 ± 1.8 g) were exposed to five concentrations of hexachloroethane (0.34, 0.67, 0.97, 1.58, and 1.83 mg/L) in duplicate, plus controls. The 96-hr LC50 concentration was 0.84 mg/L (Table 3). Fish that lost equilibrium or were motionless on the chamber bottom were considered affected. The 96-hr EC50 concentration was also 0.84 mg/L. Tetrachloroethylene--Rainbow trout from Fattig Hatchery, Brady, Nebraska (mean standard length, 6.1 cm; mean weight, 3.2 g) were exposed to five concentrations of tetrachloroethylene (2.41, 3.69, 6.39, 11.2, and 17.3 mg/L) in duplicate, plus controls. All mortalitites occurred during the first 28 hours of exposure. The 96-hr LC50 value was 4.99 mg/L (Table 3). Fish that swam abnormally or laid motionless on the chamber bottom were considered affected. The 96-hr EC50 was 4.86 mg/L.

Tetrachloroethylene/DMF--Rainbow trout from Fattig Hatchery, Brady, Nebraska (mean standard length, 7.3 cm; mean weight, 5.9 g) were exposed to five concentrations of tetrachloroethylene (2.23, 3.53, 5.95, 11.29, and 16.43 mg/L) in duplicate dissolved in dimethylformamide (DMF), plus controls. The measured concentrations of DMF in the respective exposure chambers beginning with the lowest exposure (2.23 mg/L) were 75.8, 121.7, 220.3, 326.3, and 513.0 mg/L. The 96-hr LC50 and EC50 values were 5.84 and 5.76 mg/L, respectively. Although the results of the two tetrachloroethylene tests with and without DMF are in reasonable agreement, there was concern that the test fish in this test were unhealthy and showed symptoms of distress at the termination of the test.

Hexachlorobutadiene--Rainbow trout from Lake Mills, Wisconsin, National Fish Hatchery (mean standard length, 56 cm; mean weight 3.2 g) were exposed to five concentrations of hexachlorobutadiene (66, 96, 229, 468, and 670

µg/L) in duplicate, plus controls. Fish deaths occurred throughout the 168 hrs of exposure. The 96-hr LC50 value was 320 µg/L (Table 3). Affected fish swam erratically, lost equilibrium and laid on the chamber bottom. The 96-hr EC50 value was 140 µg/L.

Acute Toxicity - Invertebrates

Daphnia magna--The 48-hr LC50s and EC50s values, including 95% confidence intervals, for eight chlorinated aliphatic compounds are presented in Table 4.

The chlorinated ethanes increased in acute toxicity with an increase in chlorine substitution (Table 4). The LC50 values ranged from 268 mg/L for 1,2-dichloroethane to 2.9 mg/L for hexachloroethane. This trend also held for the 48-hr LC50 values obtained for 1,3-dichlorobenzene and 1,2,4-trichlorobenzene (Table 4) of 7.43 and 2.09 mg/L, respectively.

In general, feeding of the animals during acute tests had no apparent effect on toxicity, with the exception of the results with tetrachloroethylene in which feeding appeared to reduce toxicity.

Chronic Toxicity-Fish

Fathead minnow early life stage (ELS) test--Larval growth was the most sensitive indicator of toxic stress during the 32-day ELS toxicity tests (Tables 5-7). Retarded growth of larval fish is critical, and could have a very profound effect on their ability to obtain food and compete with other organisms in the natural ecosystem. Mean replicate control weights of fathead minnows varied somewhat between tests. These differences in growth were probably due to differences in the quality and quantity of food offered to the fish between tests. Because of the difficulties in standardizing quantities of live food fed to fathead minnows, such differences in growth can be expected between tests, investigators, and laboratories. Regardless

TABLE 4. ACUTE TOXICITY VALUES FOR DAPHNIA MAGNA, EXPOSED TO EIGHT CHLORINATED ALIPHATIC COMPOUNDS FOR 48 HRS.

	LC50		EC50	
	Unfed	Fed	Unfed	Fed
	(mg/l)		(mg/l)	
Chlorinated Ethanes				
Hexachloroethane	2.90[3] 2.50-3.33	2.35[1] 1.99-2.86	2.10[3] 1.82-2.45	1.81[2] 1.61-2.07
Pentachloroethane	7.32[3] 5.98-8.99	8.02[3] 6.89-9.39	4.69[3] 3.99-5.50	6.88[3] 6.07-7.85
1,1,2,2-Tetrachloroethane	62.1[2] 55.9-70.7	56.9[3] 49.9-66.3	23.0[1] 16.3-34.5	25.2[3] 22.2-28.2
1,1,2-Trichloroethane	186[3] 164-214	174[3] 154-201	80.6[1] 57.5-113	77.8[1] 56.6-107
1,2-Dichloroethane	268[2] 246-293[4]	315[3] 265-414	155[1] 137-188	183[2] 154-225
Chlorinated Benzenes				
1,3-Dichlorobenzene	7.43[3] 6.29-8.77	7.23[3] 6.14-8.50	4.23[1] 3.28-5.89	5.98[1] 4.85-9.53
1,2,4-Trichlorobenzene	2.09[3] 1.80-2.63	1.68[2] 1.52-1.85	Nd	Nd
Chlorinated Ethylenes				
Tetrachloroethylene	18.1[2] 15.5-21.8	9.09[2] 7.70-11.0	8.50[1] 7.00-11.5	7.49[2] 6.08-9.03

Nd = No determination.

[1] Binomial

[2] Moving average method

[3] Probit method

[4] 95% Confidence intervals

TABLE 5. EFFECTS OF CHLORINATED ETHYLENES, PROPANES, AND BUTADIENES ON SURVIVAL AND GROWTH OF FATHEAD MINNOWS IN 32 DAY EMBRYO-LARVAL TESTS.

Chemical Tested	Mean Chemical Concentration (ug/l)	Percent Survival	Mean Individual Wet Weight (mg)
Tetrachloroethylene	0.0 (Controls)	95	258
	500	55 (explainable)	255
	1,400	83	185**
	2,800	38**	118**
	4,100	0**	0**
	8,600	0**	0**
1,2-Dichloropropane	100 (Controls)	95	145
	6,000	92	140
	11,000	95	126*
	25,000	58**	79*
	51,000	27**	18*
	110,000	0**	0*
1,3-Dichloropropane	200 (Controls)	93	125
	4,000	98	115
	8,000	93	111
	16,000	97	98*
	32,000	98	79**
	65,000	49**	25**
Hexachlorobutadiene	0.08	100	130
	1.7	98	127
	3.2	97	125
	6.5	85	125
	13.0	53**	104**
	27.0	55**	32**

* Significantly different from controls (P = .05).

** Significantly different from controls (P = .01).

TABLE 6. EFFECTS OF CHLORINATED BENZENES ON SURVIVAL AND GROWTH OF FATHEAD MINNOWS IN 32 DAY EMBRYO-LARVAL TESTS.

Chemical Tested	Mean Chemical Concentration (µg/l)	Percent Survival	Mean Individual Wet Weight (mg)
Hexachlorobenzene	.03 (Controls)	93	170
	.31	100	159
	.66	97	172
	1.16	87	164
	2.58	97	150
	4.76[a] Saturation≈10	97	165
Pentachlorobenzene	0.5 (Controls)	88	104
	3.3	89	103
	6.7	85	111
	13.0	82	108
	27.7	76	107
	54.9[a] Saturation≈120	78	99
1,2,3,4-Tetrachlorobenzene	0.35 (Controls)	92	112
	19	83	114
	39	90	114
	110	93	102
	245	82	98
	412	60*	57
1,2,4-Trichlorobenzene	15 (Controls)	92	95
	75	83	96
	134	92	89
	304	91.5	85
	499	88	86
	1,001	62*	67*
1,4-Dichlorobenzene	19	95	101
	565	93	100
	1,040	78*	87*
	2,000	0*	0*
	4,090	0*	0*
	8,720	0*	0*
1,3-Dichlorobenzene	31 (Controls)	97	100
	304	98	99
	555	97	99
	1,000	95	102
	2,267	93	67*
	3,913	7*	10*

[a] Highest concentration that could be maintained in chambers.

* Significantly different from controls (P = .05).

TABLE 7. EFFECTS OF CHLORINATED ETHANES ON SURVIVAL AND GROWTH OF FATHEAD MINNOWS IN 32 DAY EMBRYO-LARVAL TESTS.

Chemical Tested	Mean Chemical Concentration (µg/l)	Percent Survival	Mean Individual Wet Weight (mg)
Hexachloroethane	0.9 (Control)	87.5	172
	28	67.5	188
	69	75	163
	207	82.5	121*
	608	90	38**
	1,604	0**	0**
Pentachloroethane	10.0 (Control)	85.0	218
	900	82.5	226
	1,400	77.5	147**
	2,900	92.5	95**
	4,100	45.0**	46**
	13,900	0**	0**
1,1,2,2-Tetrachloroethane	12.0 (Control)	95	191
	1,400	100	186
	4,000	95	150*
	6,800	95	144**
	13,700	12.5**	25**
	28,400	0**	0**
1,1,2-Trichloroethane	50 (Control)	100	144
	2,000	100	152
	6,000	95	140
	14,800	100	122*
	48,300	77.5**	43**
	147,000	0**	0**
1,2-Dichloroethane	300 (Control)	92	134
	4,000	95	126
	7,000	92	126
	14,000	92	134
	29,000	97	120
	59,000	90	51*

* Significantly different from controls (P = .05).

** Significantly different from controls (P = .01).

of the different feedings rates between tests, one of the most important considerations when conducting an ELS toxicity test is that all groups of fish within a test are offered similar amounts of food at each feeding. Food volumes must also be adjusted accordingly when survival in any replicate is reduced by 25, 50, or 75%. The foregoing feeding method will ensure that significant growth differences (or lack of differences) between the control and test concentrations were not due simply to poor feeding technique.

Larval survival was either equal or slightly less sensitive than growth (Tables 5-7). Daily counts of live fish during each test revealed that all reductions in survival occurred within two weeks after hatch. Replicate control survival, ranging from 80-100%, was excellent during each of the exposures (Tables 5-7).

The estimated MATC for fathead minnows exposed to hexachlorobutadiene lies between 6.5 and 13.0 µg/L, and is based on reduced larval survival and weight (Table 5). The estimated MATCs for fathead minnows exposed to 1,2-dichloropropane, 1,3-dichloropropane, and tetrachloroethylene lie between 6,000 and 11,000 µg/L, 8,000 and 16,000 µg/L, and 500 and 1,400 µg/L, respectively; and are based on reduced larval weight (Table 5).

The effects of chlorinated benzenes on ELS are presented in Table 6. Hexachlorobenzene and pentachlorobenzene were not toxic near saturation, 4.76 µg/L and 54.9 µg/L respectively, therefore no estimate of MATC could be made. The estimated MATC for 1,2,3,4-tetrachlorobenzene lies between 245 and 412 µg/L based on survival. The MATC ranges for 1,2,4-trichlorobenzene (499 to 1,001 µg/L) and 1,4-dichlorobenzene (565 to 1,040 µg/L) are based on survival and growth. The estimated MATC for 1,3-dichlorobenzene was between 555 and 1,040 µg/L based on reduced larval growth (Table 6).

All estimated MATCs for chlorinated ethanes were based on wet weight data (Table 7). These MATCs are as follows: hexachloroethane (69-207 µg/L; pentachloroethane (900-1,400 µg/L); 1,1,2,2-tetrachloroethane (1,400-4,000 µg/L); 1,1,2-trichloroethane (6,000-14,800 µg/L); and 1,2-dichloroethane (29,000-59,000 µg/L).

Results obtained from the preceding ELS test method evaluations and the estimated MATCs derived from these evaluations demonstrate the usefulness and consistency of the ELS toxicity test procedures for fathead minnows currently being adopted as standards by the U.S. EPA and ASTM. These ELS test methods produced good replication; and when used to predict long-term chronic toxicity, will provide an economical means to (1) develop water quality criteria and (2) screen large numbers of single chemicals, complex effluents, or aqueous mixtures containing potentially hazardous chemicals.

Bioconcentration factors--The most readily bioaccumulated chemical in Table 8 was hexachlorobenzene and the least bioaccumulated was 1,1,2,2-tetrachloroethane. The bioconcentration potential of both the ethane and the benzene groups was directly related to the number of chlorine atoms on the molecule as shown in Table 8 by the calculated BCF values (C_F/C_W).

Chronic Toxicity - Invertebrates

Daphnia 28-day tests--The 28-day no observable effect concentrations (NOEC) were determined for Daphnia magna with three different groups of chemicals (Table 9). The chronic NOEC based on growth were identical to those based on reproduction for 1,3-dichlorobenzene, 1,2,4-trichlorobenzene and tetrachloroethylene, but varied somewhat for 1,2-dichloroethane and 1,1,2-trichloroethane. The toxicity generally increased with increasing

TABLE 8. BIOCONCENTRATION FACTORS DETERMINED FOR TEN CHLORINATED ALIPHATIC COMPOUNDS IN FATHEAD MINNOWS EXPOSED FOR 32 DAYS.

Chemical	BCF	Log BCF
Hexachloroethane	756	2.88
Pentachloroethane	62	1.79
1,1,2,2-Tetrachloroethane	7	0.87
Tetrachloroethylene	74	1.87
Hexachloro-1,3-butadiene	6988	3.84
Hexachlorobenzene	23391	4.37
1,2,3,4-Tetrachlorobenzene	2567	3.41
1,2,4-Trichlorobenzene	398	2.60
1,3-Dichlorobenzene	97	1.99
1,4-Dichlorobenzene	112	2.05

TABLE 9. CHRONIC EFFECT/NO OBSERVED EFFECT CONCENTRATION RANGES[1] FOR *DAPHNIA MAGNA* BASED ON REPRODUCTIVE SUCCESS AND GROWTH DURING 28 DAY TESTS

Compound	Chemical Concentration mg/l ($\bar{X}$±S.D.)	Number of Young Produced ($\bar{X}$±S.D.)	Length (mm) of Adults ($\bar{X}$±S.D.)
1,1,2,2-Tetrachloroethane	0.0 (Controls)	162 ± 49	No Data
	0.419 ± .036	84 ± 50	No Data
	0.859 ± .085	69 ± 39	No Data
	1.71 ± 17	71 ± 40	No Data
	3.43 ± .39	78 ± 37	No Data
	6.85 ± .90	78 ± 18	No Data
	14.4 ± 1.4	23 ± 5**	No Data
1,1,2-Trichloroethane	0.0 (Controls)	150 ± 42	4.1 ± .2
	1.72 ± .16	95 ± 53	3.9 ± .2
	3.40 ± .29	132 ± 57	3.8 ± .2
	6.35 ± .52	146 ± 55	4.1 ± .2
	13.2 ± 1.7	163 ± 59	4.0 ± .2
	26.0 ± 2.2	114 ± 31	3.9 ± .2*
	41.8 ± 3.0	11 ± 4**	3.9 ± .2*
1,2-Dichloroethane	0.0 (Controls)	164 ± 45	3.9 ± .3
	10.6 ± 0.8	128 ± 37	3.9 ± .2
	20.7 ± 1.7	88 ± 51*	3.8 ± .2
	41.6 ± 2.4	54 ± 24**	3.6 ± .2
	71.7 ± 4.8	43 ± 22**	3.4 ± .2**
	94.4 ± 5.5	19 ± 21**	3.1 ± .4**
	137.0 ± 9.0	-	2.3 ± .1**
1,2,4-Trichlorobenzene	0.0 (Controls)	166 ± 51	3.9 ± .2
	0.018 ± .003	151 ± 60	4.2 ± .2
	0.039 ± .005	159 ± 38	3.9 ± .1
	0.079 ± .011	157 ± 25	3.7 ± .1
	0.162 ± .028	125 ± 27	3.6 ± .5
	0.363 ± .056	107 ± 30	3.6 ± .2
	0.694 ± .140	32 ± 20**	3.0 ± .2**
1,3-Dichlorobenzene	0.0 (Controls)	165 ± 23	4.2 ± .1
	0.044 ± .012	167 ± 34	4.4 ± .1
	0.102 ± .023	178 ± 30	4.3 ± .1
	0.182 ± .039	212 ± 37	4.5 ± .2
	0.373 ± .053	137 ± 46	4.1 ± .2
	0.689 ± .156	190 ± 39	4.3 ± .2
	1.45 ± .28	93 ± 30**	3.5 ± .2**

TABLE 9. (Continued)

Compound	Chemical Concentration mg/l ($\bar{X}$±S.D.)	Number of Young Produced ($\bar{X}$±S.D.)	Length (mm) of Adults ($\bar{X}$±S.D.)
Tetrachloroethylene	0.0 (Controls)	154 ± 47	3.9 ± .2
	0.75 ±.036	165 ± 45	4.1 ± .2
	0.159 ± .085	111 ± 76	3.9 ± .4
	0.254 ± .094	169 ± 46	4.0 ± .2
	0.505 ± .250	169 ± 43	4.1 ± .1
	1.11 ± .480	58 ± 26**	3.6 ± .1**
	1.75 ± 1.10	0	0

[1] Chronic ranges comparable to MATCs for fathead minnows.

* Significant difference (P = .05).

** Significant difference (P = .01).

chlorination. Chlorinated ethanes were less toxic chronically, than the chlorinated benzenes and ethylenes tested (Table 9).

Evaluation of fathead minnow model to detect carcinogenesis--Recent studies with fish have indicated that certain trout strains are thousands of times more sensitive than mammals to a selected carcinogen administered in the diet (Sinnhuber et al., 1977). Further investigations have shown that the exposure of trout embryos to low ppm concentrations of a known carcinogen for one hour will produce tumors in the juvenile fish (Wales et al., 1978). The purpose of this project was to examine the possibility of using the fathead minnow to screen volatile organic chemicals found in drinking water chemicals for potential carcinogenicity.

Surviving fathead minnows from 30-day ELS exposures to organic chemicals were weighed alive at the termination of each test. Control fish and fish from the highest toxicant concentration which showed no effect were moved to freshwater aquaria. These two groups of fish (12-54 fish per group) were held until they reached sexual maturity (5-6 mos.). They were then weighed, sacrificed and examined grossly with the dissecting microscope. Special attention was given to the liver and kidney of each fish, noting general appearance and/or any abnormalities, e.g. color, texture, nodules.

Liver and kidney tissue will be prepared for histological examination by fixation in neutral buffered formalin, dehydration in graded alcohol dilutions, and embedding in JB-4 plastic. Sections 3-5μ thick were cut, placed on slides and stained with methylene blue-Azure II-basic fuchsin.

These sections will be examined microscopically for tumors and/or abnormal cells. A significant increase in tumor incidence above that seen in control animals could red flag the chemical being tested for further in-depth exposure studies.

Histological studies--All fish from the 14 exposures (Table 10) have been examined grossly and fixed in neutral buffered formalin. Twenty fish (10 controls and 10 exposed) were chosen at random from each of the 1,1,2,2-tetrachloroethane and hexachlorobenzene exposures and the livers and kidneys were dissected out and embedded in JB-4 plastic. Tissues from the 1,1,2,2-tetrachloroethane exposure have been sectioned, stained and examined microscopically.

Because of the carcinogenicity status of the ethane chemical group, fish from those exposures will be chosen next for histological examination.

TABLE 10. CHEMICALS TESTED IN FATHEAD MINNOW CARCINOGENESIS STUDY.

Chemical	Number of Fish		Carcinogen Status
	Control	Exposed	
1,2-dichloroethane (ethylene dichloride)	48	48	NCI +rat, mouse
1,1,2-trichloroethane (vinyl trichloride)	**	**	NCI +mouse
1,1,2,2-tetrachloroethane (acetylene tetrachloride)	25	54	NCI +mouse
Pentachloroethane	21	21	Currently being tested by NCI
Hexachloroethane	13	16	NCI +mouse; Being re-tested
Hexachlorobutadiene	50	40	EPA TSCA Inventory
1,3-dichlorobenzene (isomer of 1,4-dichlorobenzene)	13	17	*
1,4-dichlorobenzene (p-dichlorobenzene)	24	24	Currently being tested by NCI
1,2,3,4-tetrachlorobenzene	16	24	*
Pentachlorobenzene	15	14	*
Hexachlorobenzene	41	44	*
Tetrachloroethylene (Perchloroethylene)	27	12	NCI +mouse; Being re-tested
1,2-dichloropropane (Propylene dichloride)	51	50	Currently being tested by NCI
1,3-dichloropropane	37	53	EPA TSCA Inventory

* Benzene is a human suspect carcinogen; animal studies are inadequate.

** 56 fish total; control and exposed fish were mixed in aquarium.

SUMMARY AND CONCLUSIONS

These data have been used in a number of important ways from criteria documents and the structure-activity data base on aquatic toxicology to an evaluation of the use of aquatic organisms in a screening program to serve as an early warning system for higher animals including man. Many of these chemicals have been detected in surface and subsurface drinking water supplies of major cities, but at concentrations well below those causing obvious acute toxic effects on higher animals or man. There was, however, concern over the long term chronic effects of these chemicals continually available to a population in the drinking water supply. It has long been known that aquatic animals are extremely sensitive to chemicals of all kinds at very low µg/L to mg/L concentrations in the aqueous environment. This knowledge led us to the position of evaluating both acute and chronic toxicity tests with several sensitive aquatic species in an effort to determine the range of sensitivities and the possible application of the data to the red flagging of chemicals, which after short inexpensive tests with selected aquatic species were shown to be extremely toxic, highly bioaccumulatable, and/or cause an increased incidence of tumors in exposed animals.

Phase I of these studies, submitted as a separate report, was designed to give us some preliminary information on the metabolic capabilities of several of the lower animals (rainbow trout, Salmo gairdneri and water flea, Daphnia magna). These studies coupled with earlier studies on MFO activity in mammals and lower animals indicate the metabolic systems to be similar qualitatively, therefore, the mechanisms leading to toxicity and neoplasia, for example, are presumed to be similar in all organisms. Hence, aquatic

animals are being used in laboratory screening and in environmental monitoring.

Phase II was involved with the acute and chronic toxicity of five classes of chlorinated organic compounds to selected fish and invertebrate animals. In addition, the bioconcentration potential of these chemicals was important in the determination of possible food-chain problems involving man.

Of the five chemical classes tested the most acutely toxic to fish was the one representative of the butadiene class hexachlorobutadiene followed in decreasing order of toxicity by the chlorinated benzenes, ethylenes, ethanes, and the propanes (Table 11). The invertebrate Daphnia magna showed the same order of sensitivity as the fish for those classes of chemicals tested.

A comparison of species sensitivities in Table 11 indicated that Daphnia was slightly more resistant than the fathead minnow, although quite similar, while the rainbow trout was considerably more sensitive than either the fathead or Daphnia except for the hexachlorobutadiene exposures.

One of the more interesting findings of the acute studies was the increased toxicity of the ethanes, benzenes and ethylenes as the number of chlorines on the molecule increased. This was true for both fathead minnows and Daphnia (Table 11).

These data indicate that either fathead minnows or Daphnia would provide essentially the same acute values for these particular chemicals. It is also true that these chemicals are not considered to be very toxic to aquatic species, since their 96-hr LC50s are one to two orders of magnitude above those environmental chemicals considered as extremely toxic.

Fifteen chronic toxicity tests with fish were also conducted on chemicals in the five chemical classes. As with the acute toxicity tests the

TABLE 11. SUMMARY OF ACUTE TOXICITY DATA FOR FATHEAD MINNOWS, RAINBOW TROUT, AND DAPHNIA.

Compound	Fathead Minnow 96-hr LC50 (mg/1)	Rainbow Trout 96-hr LC50 (mg/1)	Daphnia 48-hr LC50 (mg/1)
Chlorinated Ethanes			
Hexachloroethane	1.53	0.84	2.90
Pentachloroethane	7.30	a	7.32
1,1,2,2-Tetrachloroethane	20.30	a	62.10
1,1,2-Trichloroethane	81.70	a	186.0
1,2-Dichloroethane	117.80	a	268.0
Chlorinated Benzenes			
Hexachlorobenzene	b	b	a
Pentachlorobenzene	b	b	a
1,2,3,4-Tetrachlorobenzene	1.07	a	a
1,2,4-Trichlorobenzene	2.76	1.52	2.09
1,3-Dichlorobenzene	7.79	1.61	7.43
1,4-Dichlorobenzene	4.16	1.12	a
Chlorinated Ethylenes			
Tetrachloroethylene	13.50	4.99	18.10
1,1,2-Trichloroethylene	44.10	a	a
Chlorinated Propanes			
1,2-Dichloropropane	139.30	a	a
1,3-Dichloropropane	131.10	a	a

TABLE 11. (Continued)

Compound	Fathead Minnow 96-hr LC50 (mg/l)	Rainbow Trout 96-hr LC50 (mg/l)	Daphnia 48-hr LC50 (mg/l)
Chlorinated Butadienes			
Hexachlorobutadiene	0.10	0.32	[a]

[a] Not tested.

[b] Not toxic at saturation.

chlorinated butadiene - hexachlorobutadiene was the most toxic follwed by benzenes, ethylenes, ethanes, and propanes in order of decreasing toxicity (Table 12). Six chronic values were also determined for Daphnia and in most cases the sensitivity was similiar except for the ethanes where there seemed to be considerable variation between the fathead and Daphnia results. Again, chronic toxicity increased considerably for both species as the number of chlorines on the molecule increased (Table 12).

The bioconcentration potentials of these chemicals were determined by establishing a bioconcentration factor (BCF) (C_{Fish}/C_{Water}) for fathead minnows exposed for 32-days to each chemical during the early life-stage toxicity test. These BCFs were then compared to BCF values for other species of fish found in the literature (Table 13). In this study with the fathead minnows the benzenes bioconcentrated the most followed by hexachlorobutadiene, the ethanes, and the ethylenes. It is interesting to note again that bioconcentration also increases as the number of chlorines on the molecule increases just as toxicity increased. The literature values for other fathead minnow studies as well as bluegill and guppys all agree very closely with the BCFs generated during the 32-day early life-stage toxicity tests. This is an important finding in that it indicates age, size or species of fish has little effect on the BCF generated over a 30-day period of water exposure. Based on BCF values hexachlorobenzene, hexachlorobutadiene, and 1,2,3,4-tetrachlorobenzene are the chemicals in the group which might pose the greatest bioconcentration problem in the environment.

Phase III

A carcinogenesis model using fathead minnows was designed to establish whether or not fish might be a sensitive indicator of carcinogenesis in the environment. Previous studies of fish had indicated exposure in the low ppm

TABLE 12. SUMMARY OF FATHEAD MINNOW AND DAPHNIA CHRONIC TOXICITY DATA.

Compound	Fathead Minnow 32-day (ELS) MATC (µg/l)	Daphnia 28-day[a] Chronic (µg/l)
Chlorinated Ethanes		
Hexachloroethane	69-207	-
Pentachloroethane	900-1,400	-
1,1,2,2-Tetrachloroethane	1,400-4,000	6,850-14,400
1,1,2-Trichloroethane	6,000-14,800	13,200-26,000
1,2-Dichloroethane	29,000-59,000	10,600-20,700
Chlorinated Benzenes		
Hexachlorobenzene	4.76[b]	-
Pentachlorobenzene	54.9[b]	-
1,2,3,4-Tetrachlorobenzene	245-412	-
1,2,4-Trichlorobenzene	499-1,008	363-694
1,3-Dichlorobenzene	1,000-2,267	689-1,450
1,4-Dichlorobenzene	565-1,040	-
Chlorinated Ethylenes		
Tetrachloroethylene	500-1,400	505-1,110
1,1,2-Trichloroethylene	-	-
Chlorinated Propanes		
1,2-Dichloropropane	6,000-11,000	-
1,3-Dichloropropane	8,000-16,000	-

TABLE 12. (Continued)

Compound	Fathead Minnow 32-day (ELS) MATC (ug/l)	Daphnia 28-day[a] Chronic (ug/l)
Chlorinated Butadienes		
Hexachlorobutadiene	6.5-13.0	-

[a] Effect - no effect concentrations.

[b] Saturation - no effects noted.

TABLE 13. A COMPARISON OF BIOCONCENTRATION FACTORS FOR CHEMICALS TESTED IN PRESENT STUDY IN FATHEAD MINNOWS VS. OTHER SPECIES OF FISH IN OTHER STUDIES.

Chemicals	Present Study Fathead minnows[a]		Literature Values[b]		
	BCF	Log BCF	Fathead Minnow[c]	Bluegill[c]	Guppy[d]
Chlorinated Ethanes					
Hexachloroethane	757	2.85	-	138	-
Pentachloroethane	62	1.78	-	68	-
1,1,2,2-Tetrachloroethane	7	0.91	-	8	-
Chlorinated Ethylenes					
Tetrachloroethylene	75	1.79	-	49	-
Chlorinated Butadienes					
Hexachloro-1,3-butadiene	6,988	3.84	-	-	-
Chlorinated Benzenes					
Hexachlorobenzene	23,391	4.37	21,878	-	14,454
1,2,3,4-Tetrachlorobenzene	2,567	3.41	-	1,820	3,631
1,2,4-Trichlorobenzene	398	2.60	1,698	-	646
1,3-Dichlorobenzene	97	1.99	-	66	-
1,4-Dichlorobenzene	112	2.05	-	60	91

[a] 32-day exposure ELS toxicity test.

[b] G. Veith, D. Call, and L. Brooke, (In preparation).

[c] 30-day old fish exposed for 30-days.

[d] Adult fish exposed for 30-days.

range to developing embryos was sufficient to induce liver tumors (Wales et al., 1978). The present studies on 14 chemicals representing 5 classes of organic chemicals indicated that gross tumors in the liver or kidney were not present 4 months after hatching, however, a microscopic work up on the ethane group (many of which are known carcinogens) is underway now and will provide more information on the usefulness of this approach as an early warning experiment for environmental carcinogenesis.

The acute toxicity tests run with both fish and invertebrates established a relative order of toxicity of the individual chemicals that was identical to the order seen in the more sensitive chronic exposures. Therefore, the short 4-day 96-hr LC50 fish exposures or the 48-hr Daphnia exposures could be used to establish a priority list of chemicals found to occur in drinking water to initially concentrate the more expensive chronic testing on the more toxic materials.

The Daphnia acute test would be better than a fish acute, since it is only 48-hrs long and does not require the more difficult flow-through system required for a fish 96-hour LC50, yet it gives the same relative order of chemical sensitivity (Tables 11 and 12).

Early life-stage toxicity tests with fish or 28-day Daphnia chronics would provide the most sensitive tests for drinking water; however, the fish exposures would also allow the determination of a BCF and the possibility of determining an increased incidence of tumors in exposed fish, both of which would provide further information for red flagging (prioritizing) chemicals for more in-depth testing on mammals.

Since the amounts of these chemicals in drinking waters are in low µg/L amounts it would be necessary to concentrate samples for testing, since these

chemicals or groups of these chemicals would not be toxic to aquatic animals at most ambient levels now reported for U.S. drinking water supplies.

The usefulness of aquatic tests for red flagging chemicals in these particular classes in drinking water may be somewhat limited, because of their low toxicity and low ambient water concentrations. However, this approach for other more toxic chemicals has considerable promise as an early warning system for higher animals including man.

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APPENDIX A: Early Life Stage Mini-Diluter Design Manual

This manual was designed to be used as a supplemental guide for the construct of a continuous-flow mini-diluter system for toxicity testing which has been described and evaluated by Benoit et al. (1982 - see references). Additional studies conducted by Anderson (Manuscript), Carlson and Kosian (Manuscript), Spehar et al. (1980), and Benoit et al. (1982 - see references) have demonstrated the usefulness of this test system and have also illustrated the type of data one can expect to obtain with young fish and macroinvertebrates.

The following text on glass cutting, assembly, and equipment was taken from Lemke et al. (1978). This information was included to familiarize the reader with techniques currently used at the U.S. EPA Environmental Research Laboratory-Duluth.

Glass Construction Equipment

Recommended equipment for diluter construction includes: sharp glass cutters, a glass cutting table, a glass saw, a set of glass drills, designed for use on a standard heavy duty drill press, and a power stopper borer.

Sharp glass cutters are needed to obtain straight, smooth cuts to prevent leaks. An optional piece of equipment is the glass cutting board, similar to those used by hardware stores to cut window panes. One style is available from Fletcher Terry Co., Bristol, Conn. 06010. A large flat surface and a good straight edge may be substituted. The glass saw is used for cutting glass tubing and is generally useful for a variety of cutting purposes. It is used to make cut ends on tubing, both square and angled, as required during diluter construction. A rolling table model, such as the Model C manufactured by Pistorius Machine Co., Hicksville, N.Y. 11801, is desirable, but if only diluter and other glass tubing is to be cut, their Model CC12, which has a tilting table, is satisfactory. The glass drills are

necessary to drill holes in the various glass cells and are listed as diamond impregnated tube drills in the catalog of Sommer and Maca, Glass Machinery Co., 5501 W. Ogden Ave., Chicago, Ill. 60650. These drills are relatively expensive, but enable the diluter builder to also drill drain holes in aquaria and test chambers. The drill press can be of any type, but should be sturdy and vibration free. Turpentine is an excellent cooling lubricant to use when drilling glass, and is recommended over water.

The boring of stoppers for various parts of a diluter is time consuming, and a power stopper-borer, such as that manufactured by E. H. Sargent Co. (Model No. S-232DT), is very useful. Some glass bending is necessary, therefore, an air-blast-type burner, such as that manufactured by Fisher Scientific Co., is very convenient. This burner enables the operator to apply sufficient heat to the tubing to allow uniform bending.

Accurate rulers and steel tapes, a micrometer for inside and outside measurement, felt marking pens, and a sufficiently large work area to prevent moving of assembled parts during construction and assembly also save time and increase efficiency.

Glass Cutting

The primary skill needed to be successful in building a diluter is the ability to cut glass with straight edges and parallel sides. A commercial glass cutting board, if well maintained, is particularly good for long cuts. A second technique is to use a large flat sturdy table and a heavy ruler or other straight edge to guide the cutter. This latter technique is faster and more versatile once mastered. All pieces should be cut with minimum tolerance. After cutting, all edges should be dulled with a stone or fine-grit sand paper to prevent hand cuts. The pieces should be cleaned by washing in a detergent solution and then rinsed thoroughly and dried.

Removal of grime is necessary to ensure good glue adhesion. Glass should be double strength (3 mm thick), but the "B" or second grade is satisfactory. Flint glass tubing is preferred to Pyrex because the lower melting point of the former makes bending and cutting the glass easier.

Glass Assembly

The most important construction material is the silicone sealant or glass glue. Dow Corning Glass and Ceramic Cement and General Electric Corporation RTV are both satisfactory. Glues that are listed as dish-water safe are preferable so that cleaning the assembled diluter with hot water will not cause the joints to fail. Disposable 10- or 15-ml plastic syringes with enlarged bores in the tips for faster application are useful for applying a thin bead of glue as needed and can be used with one hand (Figure 1). Application with the original collapsible tube requires two hands to maintain a steady and constant flow of glue from tube to the edges of the glass. If the bead of glue is too thin, any irregularities in glass cutting will not be filled by glue and will leak.

Lines are drawn to show the location of the cell dividers during assembly. A wax pencil or felt pen can be used. It is important to remember during assembly, however, that these marked surfaces should be on the outside of the cells so that glue adhesion is not affected by these lines. Waxed or other paper is placed on the table top to catch any excess glue. The paper can be removed easily after the glue has dried. Slight pressure at all glued joints distributes the glue, helps prevent leaks, and places the glass surfaces in closer contact. To ensure against leaks, a pencil eraser or rounded wooden dowel may be used to spread the freshly applied, excess glue along each seam. Use care to avoid moving the glass. After all cells have

dried overnight, they should be tested for leaks after plugging the drilled holes.

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Carlson, A. R., and P. A. Kosian. Toxicity and bioconcentration of several chlorinated benzenes in fathead minnows. U.S. Environ. Prot. Agency, Environmental Research Laboratory-Duluth, Duluth, Mn. 55804. (Manuscript).

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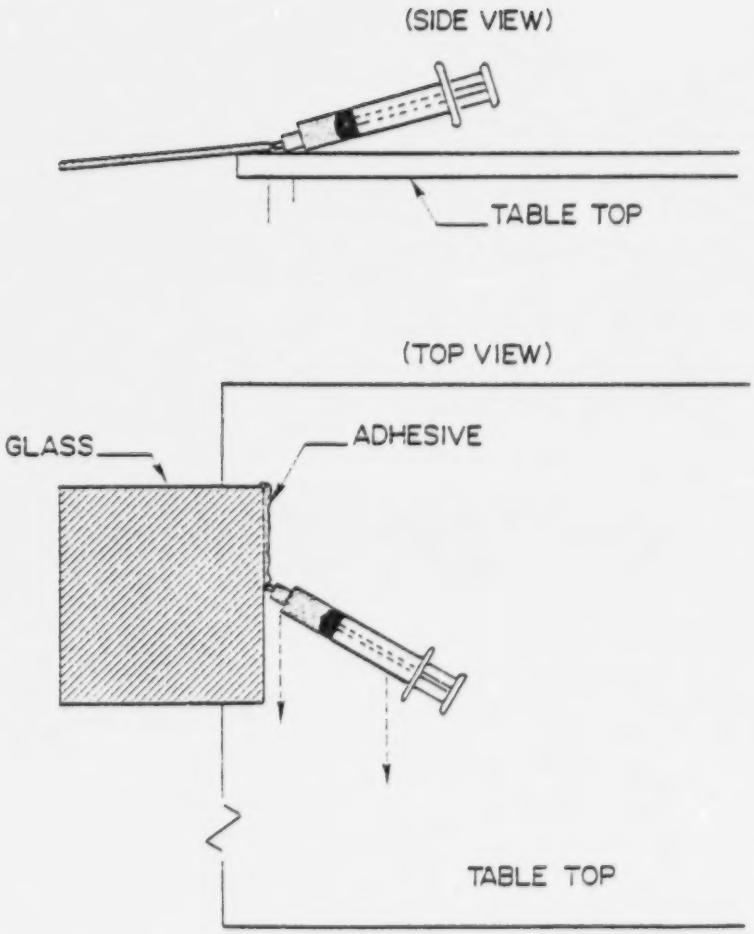


Figure 1. Glue-application system. Syringe tip to be bored out to approximately 4 mm to give sufficient bead size.

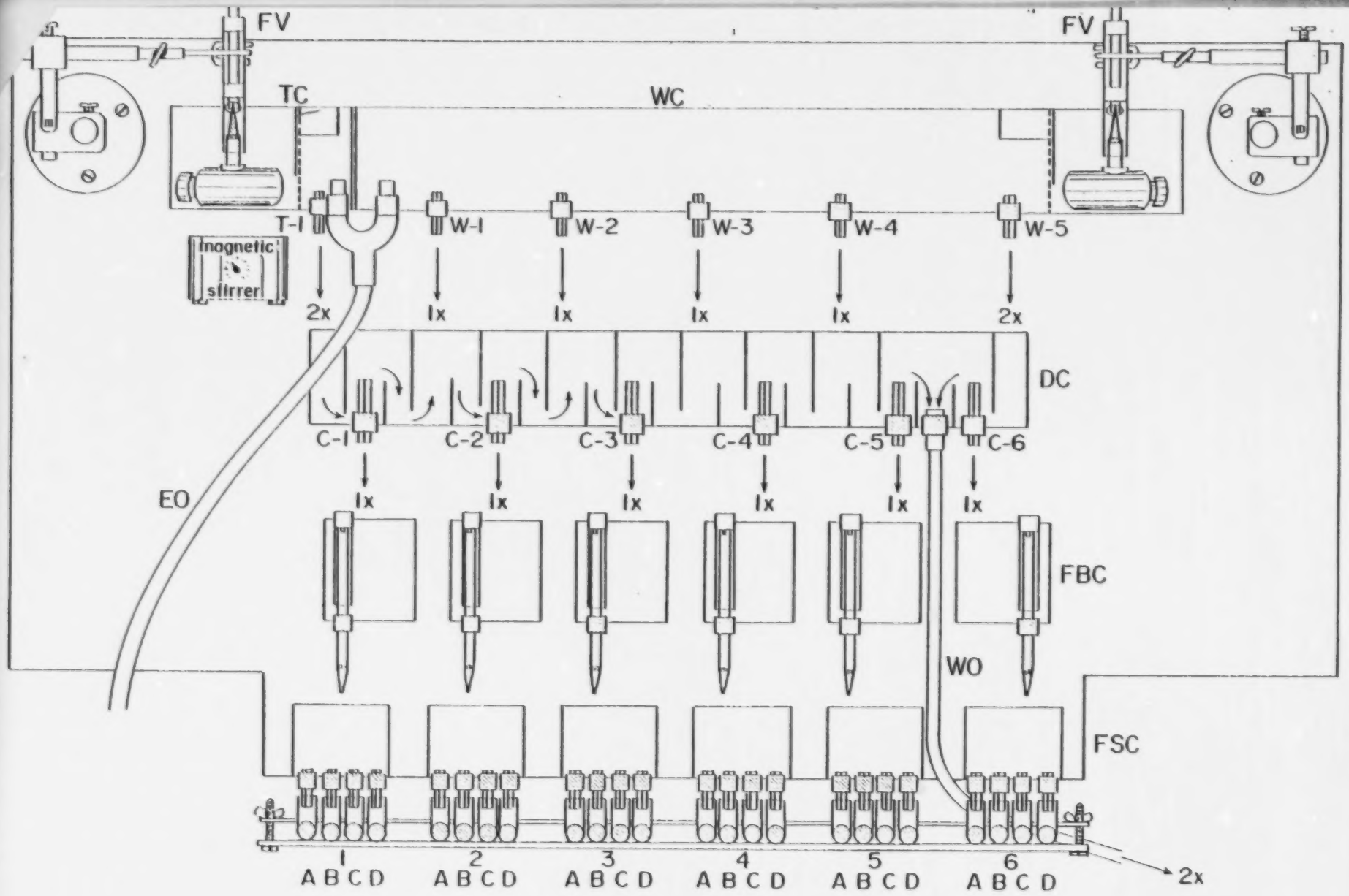


Figure 2. Schematic drawing and flow pattern of continuous flow mini-diluter. Legend: (C), concentration flow tube; (DC), dilution cell; (EO), emergency outlet; (FBC), flow booster cell; (FSC), flow splitter cell; (FV), float valve; (T), toxicant flow tube; (TC), toxicant cell; (W), water flow tube; (WC), water cell; (WO), water outlet; (1X), one volume; (2X), two volumes.

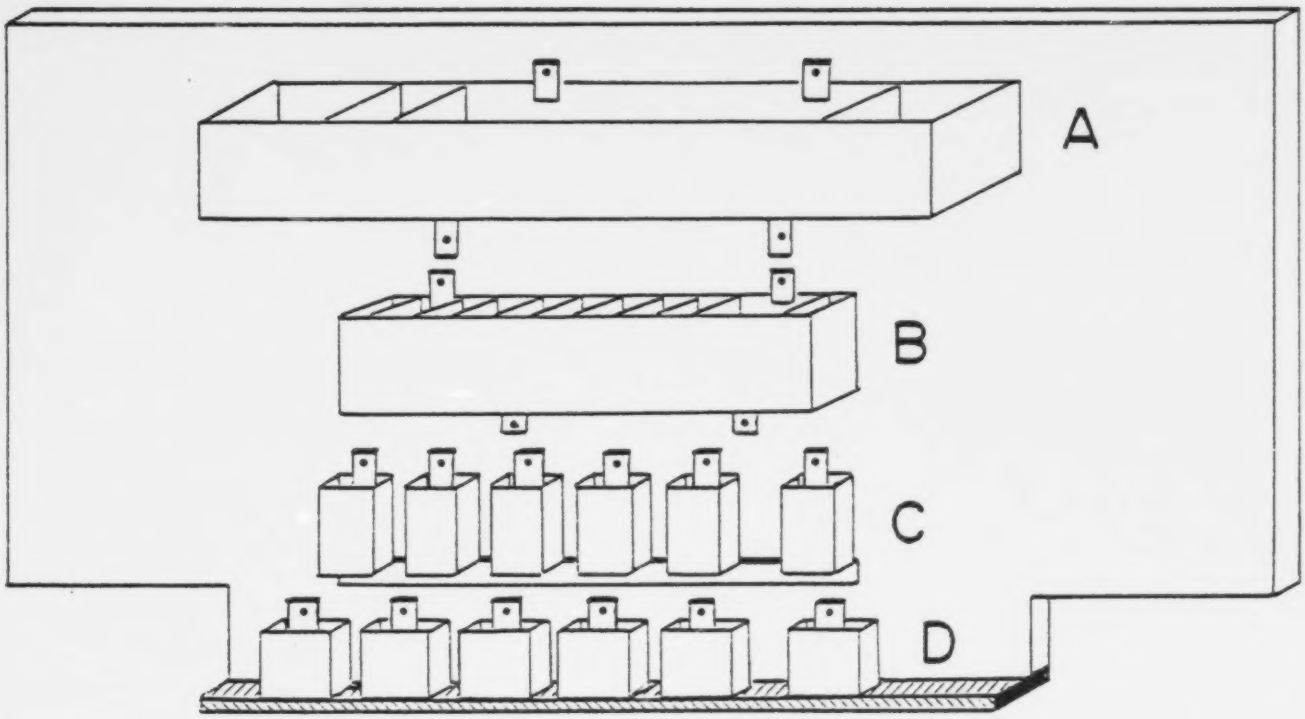


Figure 3. Diluter cells attached to back board: (A), toxicant and water cell; (B), dilution cell; (C), flow booster cells; (D), flow splitter cells.

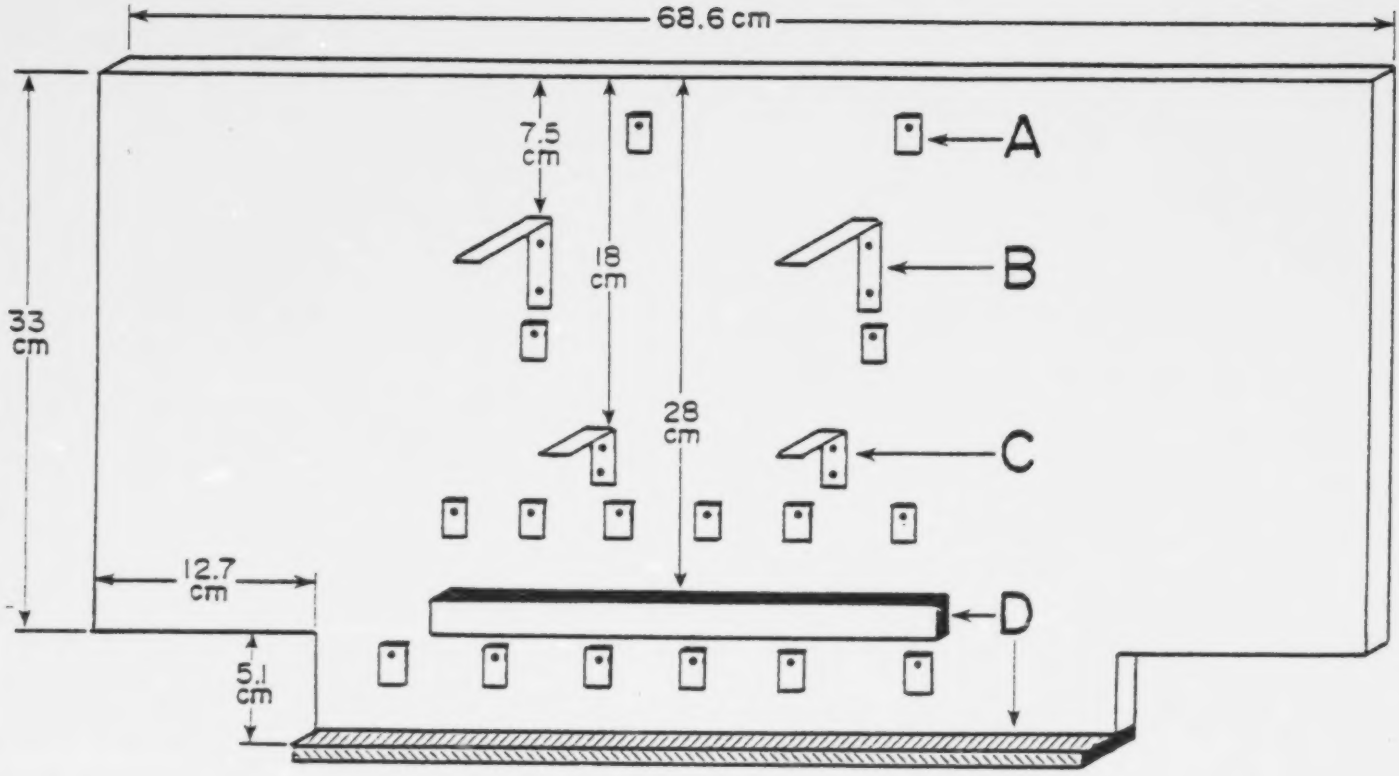


Figure 4. Diluter back board made of 1.9 cm exterior plywood: (A), plastic storm window clips; (B), metal shelf bracket (5.1 cm); (C), metal shelf bracket (3.8 cm); (D), sheet plastic, plywood or metal shelf (0.3 cm wide).

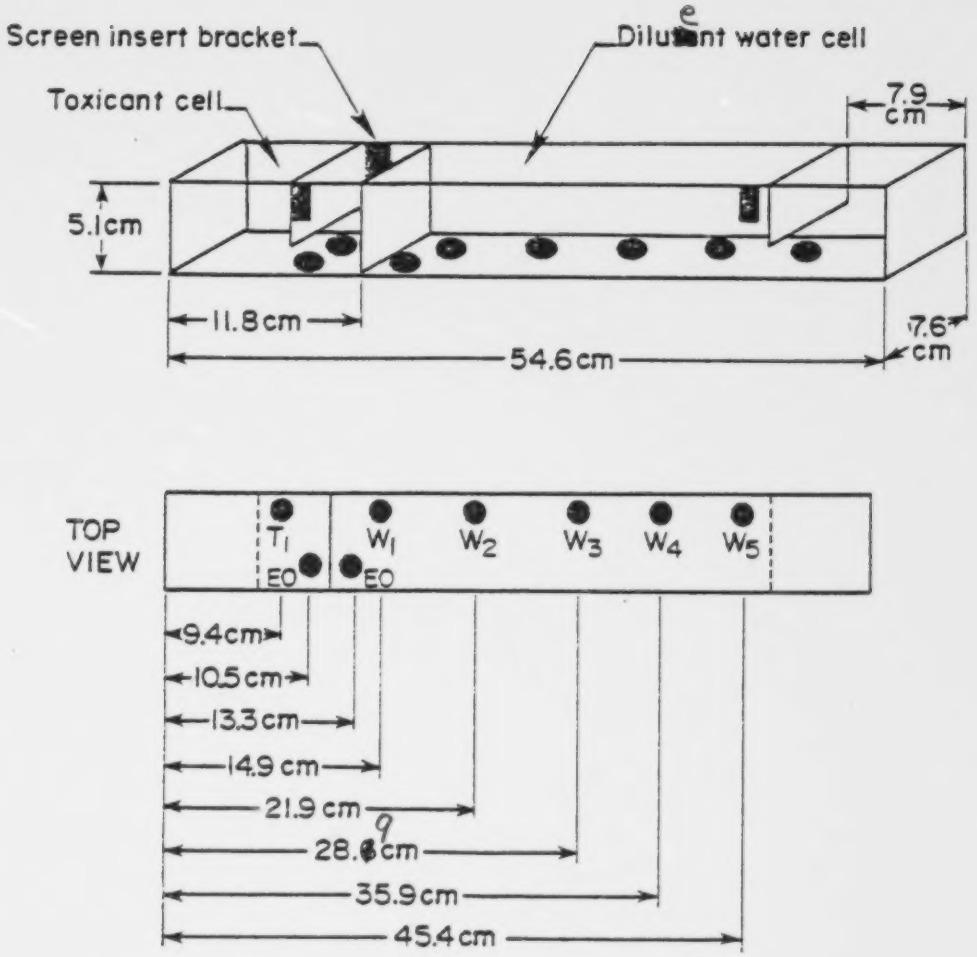


Figure 5. Toxicant and water cell. A constant depth is maintained at 3 cm in each cell to obtain the prescribed flow rates. Arrows showing location of drilled holes denote distance from left edge of glass to center of hole. (T, toxicant; W, water; EO, emergency outlet - 1.4 cm holes).

Capillary flow tube	Adjusted flow rate	Size (ID)	Length	Stopper	Drilled hole
T-1 and W-5	100 mL/min	2 mm	3 cm	#0	1.4 cm centered 1.6 cm from edge
W-1 through W-4	50 mL/min	1.5 mm	2.5 cm	#0	1.4 cm centered 1.6 cm from edge

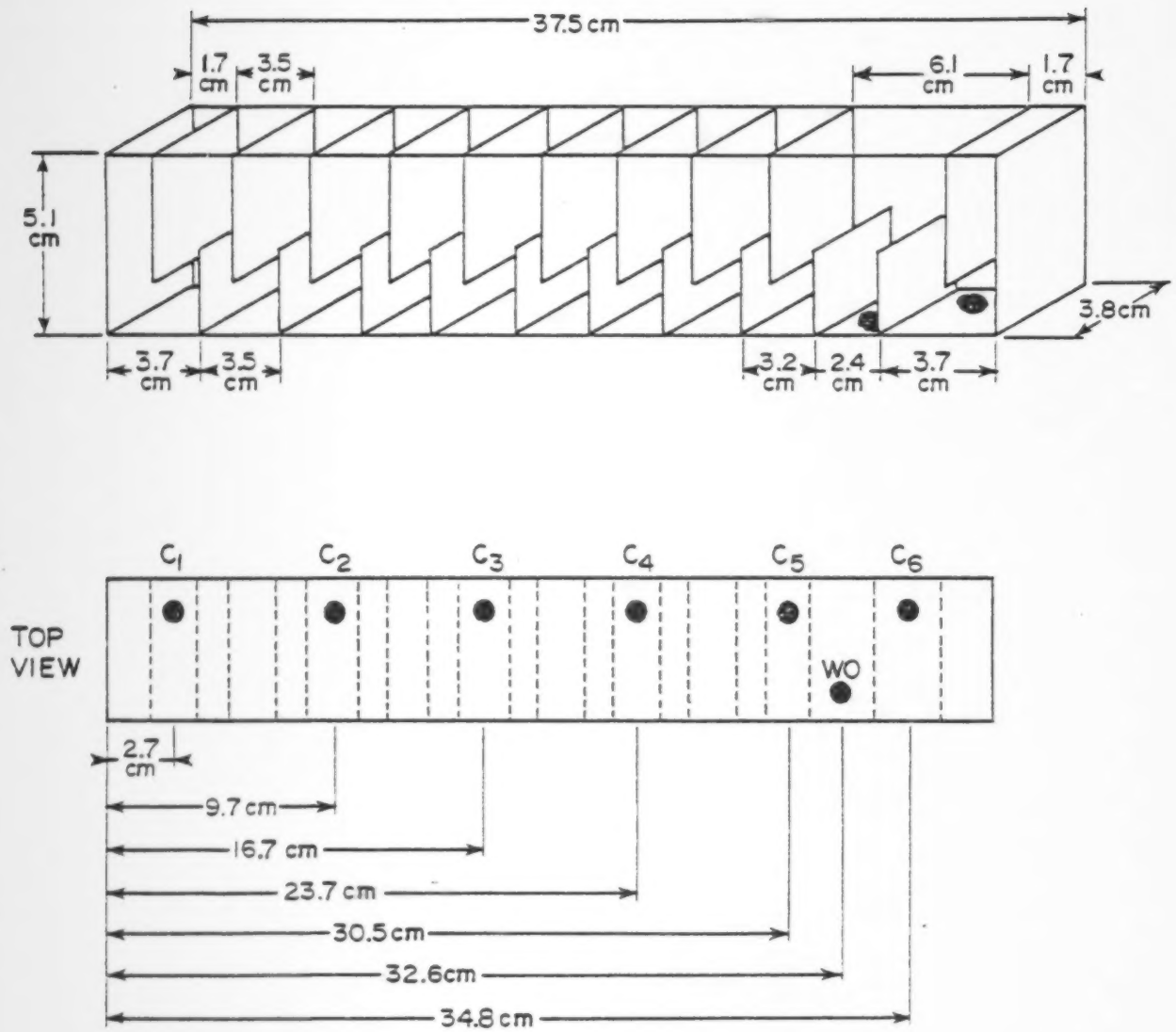
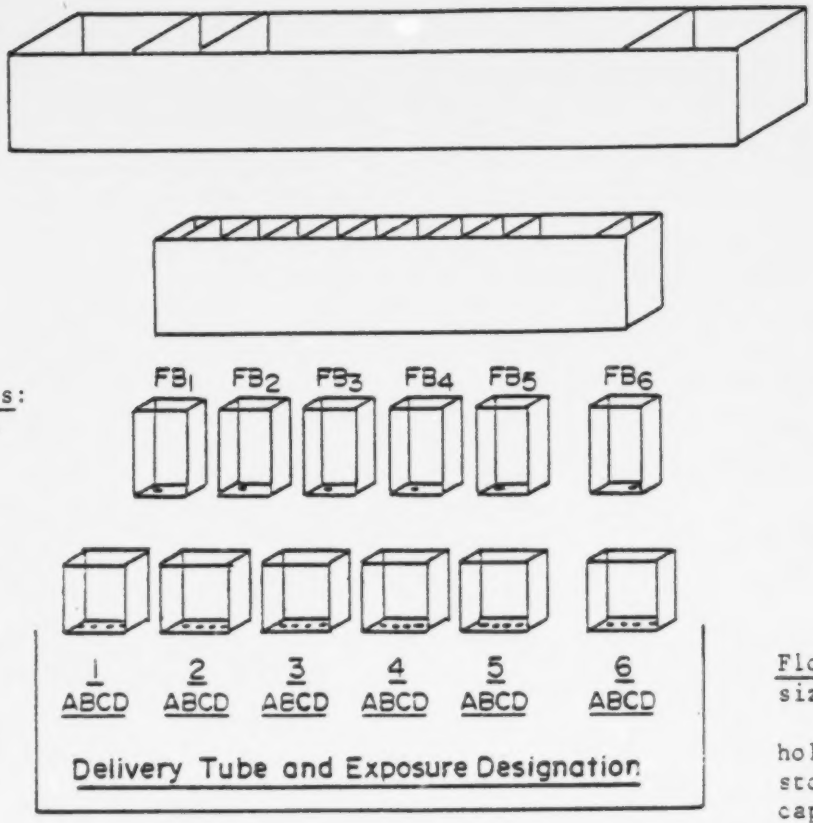


Figure 6. Dilution cell. Arrows showing location of drilled holes denote distance from left edge of glass to center of hole. (C, concentration; WO, water outlet - 1.4 cm hole).

Capillary flow tube	Adjusted flow rate	Size (ID)	Length	Stopper	Drilled hole
C-1 through C-6	50 mL/min	1.5 mm	3.5 cm	#0	1.4 cm centered 1.3 cm from edge



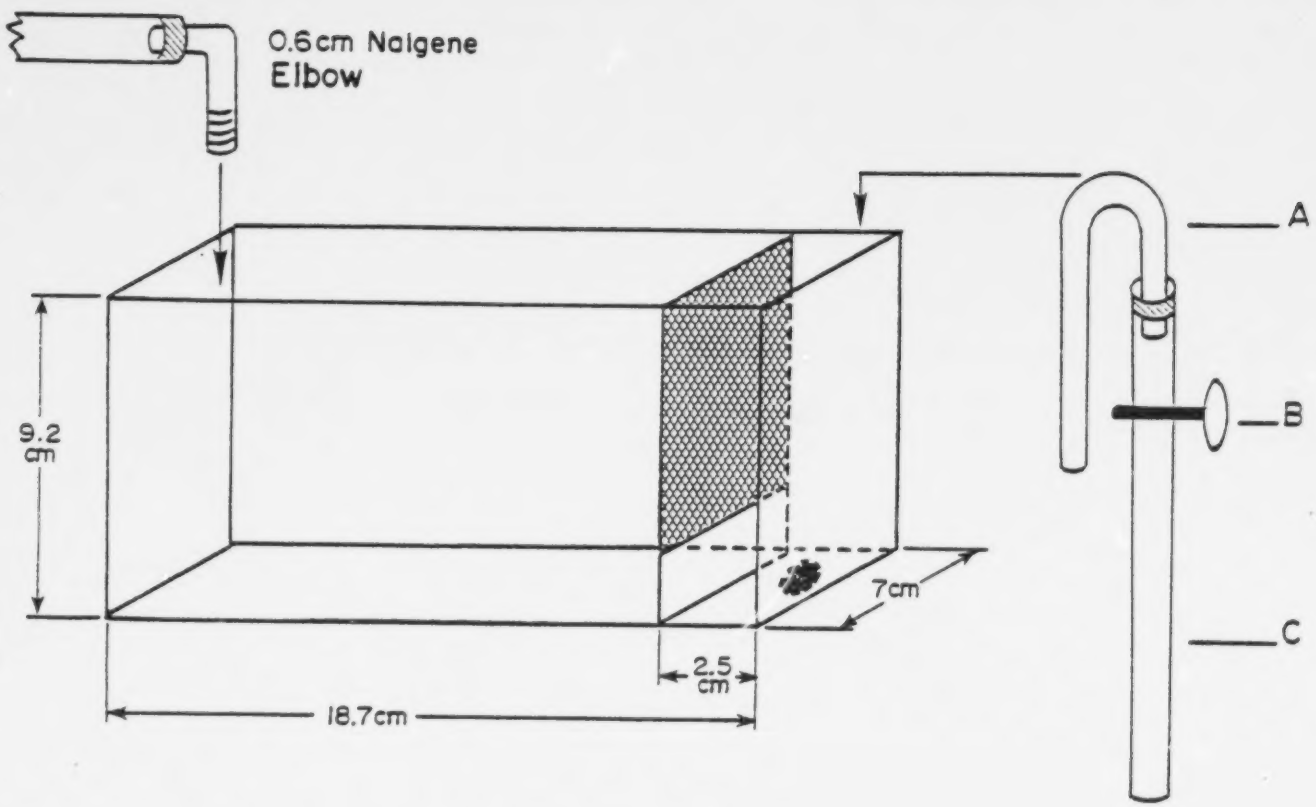
Flow booster cells:
size, 2.5 x 4.5 x 5.4 cm
hole, 1.3 cm
stopper, #00

Flow splitter cells:
size, 2.5 x 5.7 x 4.8 cm
hole, 1 cm
stopper, #000
capillary flow tube:
size, 1.5 mm (ID),
length, 2.5 cm

Flow booster siphon

siphon standpipe:	6 mm (OD) glass tube cut 8.3 cm long with glass saw notches (3 mm deep) on upper end and lower end tapered.
siphon sleeve:	11 mm (OD) glass tube cut 3.8 cm long with a #000 stopper.

Figure 7. Flow booster cells with siphon, and flow splitter cells. The lower ends of all capillary flow splitter tubes fit loosely into 1.3 cm (OD) Nalgene elbows which are attached with Bev-A-Line® tubing to 6 mm (OD) glass delivery tubes.



Water depth, 4.5 cm
Drain hole, 1.4 cm
Stopper, # 0
Standpipe, 6mm (OD) glass tube
cut 7cm long
Stainless steel screen, 40 mesh,
.010 wire

A, 6mm (OD) glass tube
B, pinch clamp
C, flexible Teflon tube

Figure 8. Exposure chamber with 6 mm (OD) glass delivery tube and water sampling siphon.

Table 1. Dimensions and Number of Double Strength Glass Pieces Needed[1] to Construct One Mini-Diluter and 24 Exposure Chambers As Shown in Figures 2-8.

Toxicant and Water Cell Unit:

bottom - 7.6 x 54.6 cm (1)
sides - 5.1 x 54 cm (2)
ends - 5.1 x 7.6 cm (2)
full divider - 5.1 x 7 cm (1)
partial divider - 3.8 x 7 cm (2)
screen holder - 3.2 x 1.3 cm (4)
stainless steel screen (20 mesh, .016 wire) - 5.1 x 7 cm (2)

Dilution Cell:

bottom - 3.8 x 37.5 cm (1)
sides - 5.1 x 37.5 cm (2)
ends - 3.8 x 5.4 cm (2)
upper dividers - 3.2 x 4.5 cm (10)
lower dividers - 2.5 x 3.2 cm (10)

Flow Booster Cells:

bottom - 2.5 x 4.5 cm (6)
sides - 3.8 x 5.1 cm (12)
ends - 2.5 x 5.1 cm (12)

Flow Splitter Cells:

bottom - 2.5 x 5.7 cm (6)
sides - 4.5 x 5.1 cm (12)
ends - 2.5 x 4.5 cm (12)

Exposure Chambers:

bottom - 7 x 18.8 cm (24)
sides - 8.9 x 18.1 cm (48)
ends - 7 x 8.9 cm (48)
divider - 1.9 x 6.4 cm (24)
stainless steel screen (40 mesh, .010 wire) - 6.4 x 7 cm (24)

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[1] If glass drilling is not convenient, the bottoms of each diluter cell and exposure chamber may be made from #316 stainless steel (3 mm thick).

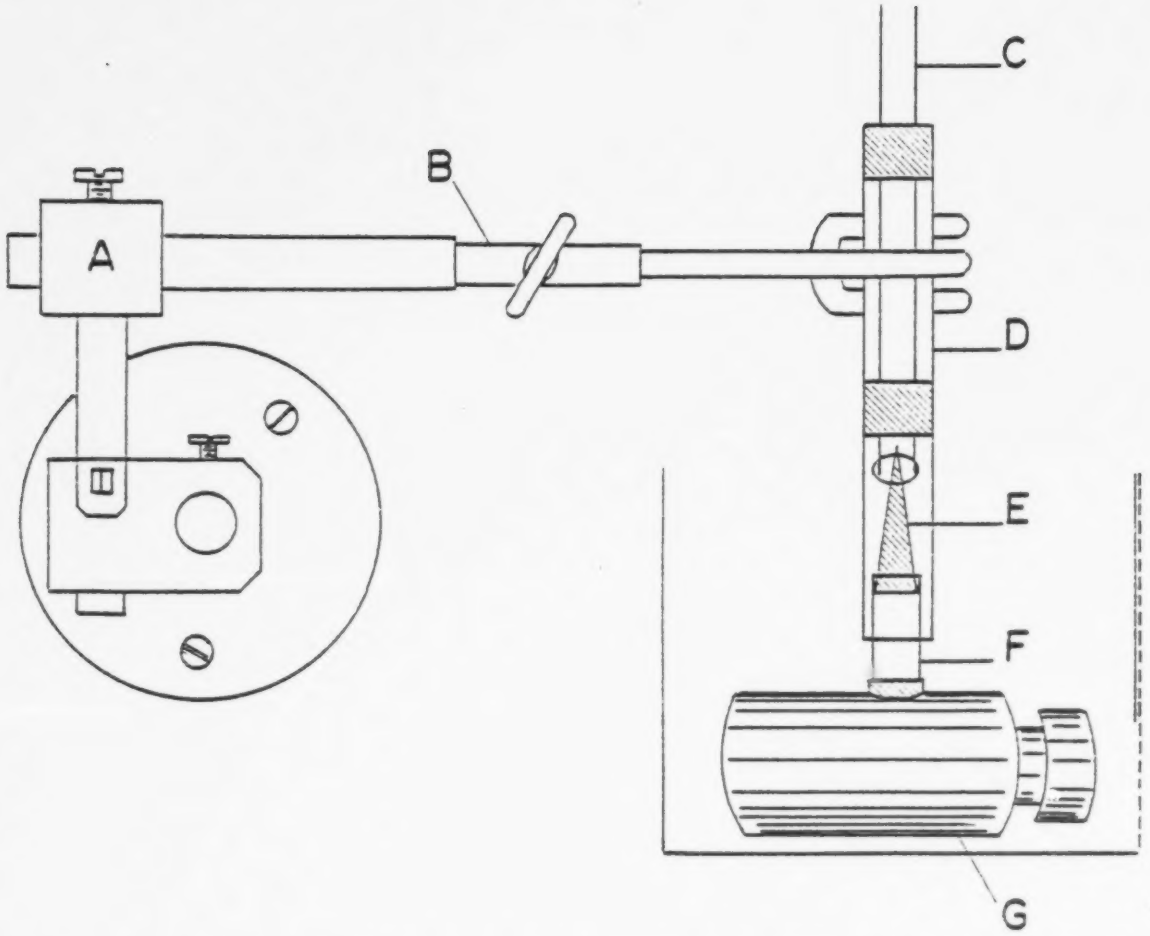

Figure 9. Diluter float valve used to maintain a constant head pressure in the toxicant and water cell.
(A), aluminum Flexaframe® fittings; (B), extension clamp, medium; (C), 7 mm (OD) glass tube water inlet, 8 cm long; (D), 14 mm (OD) glass tube stationary sleeve, 10 cm long; (hole notched in each side to let water out and to clean valve orifice); (E), neoprene tapered micro-stopper; (F), 11 mm (OD) glass tube sliding sleeve, 4 cm long; (G), float made from 30 ml Nalgene® bottle with 1.4 cm hole bored in center of one side.

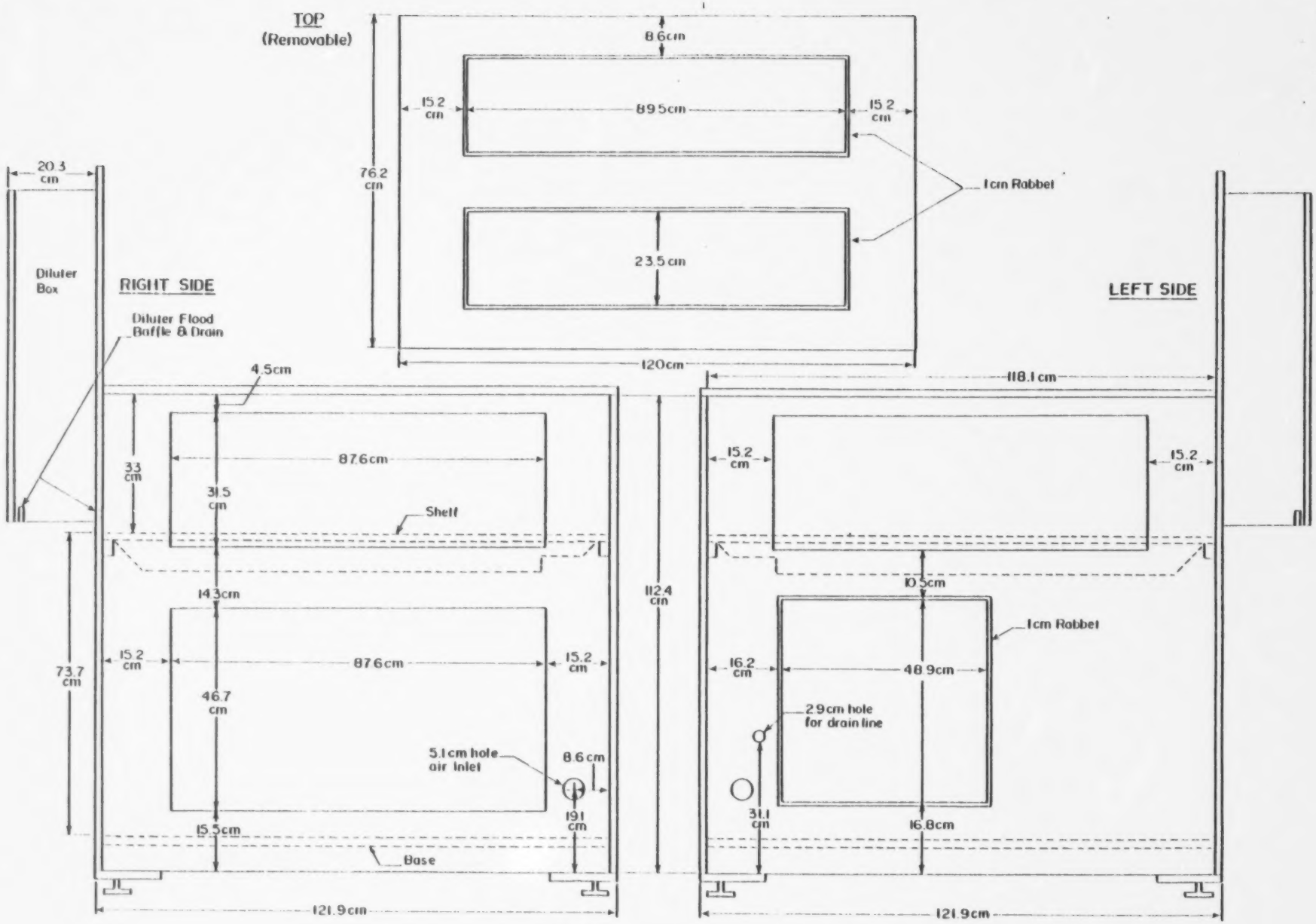

Figure 10. Vented enclosure used with the mini-diluter system to test hazardous volatile chemicals. (Diluter box and exposure box)

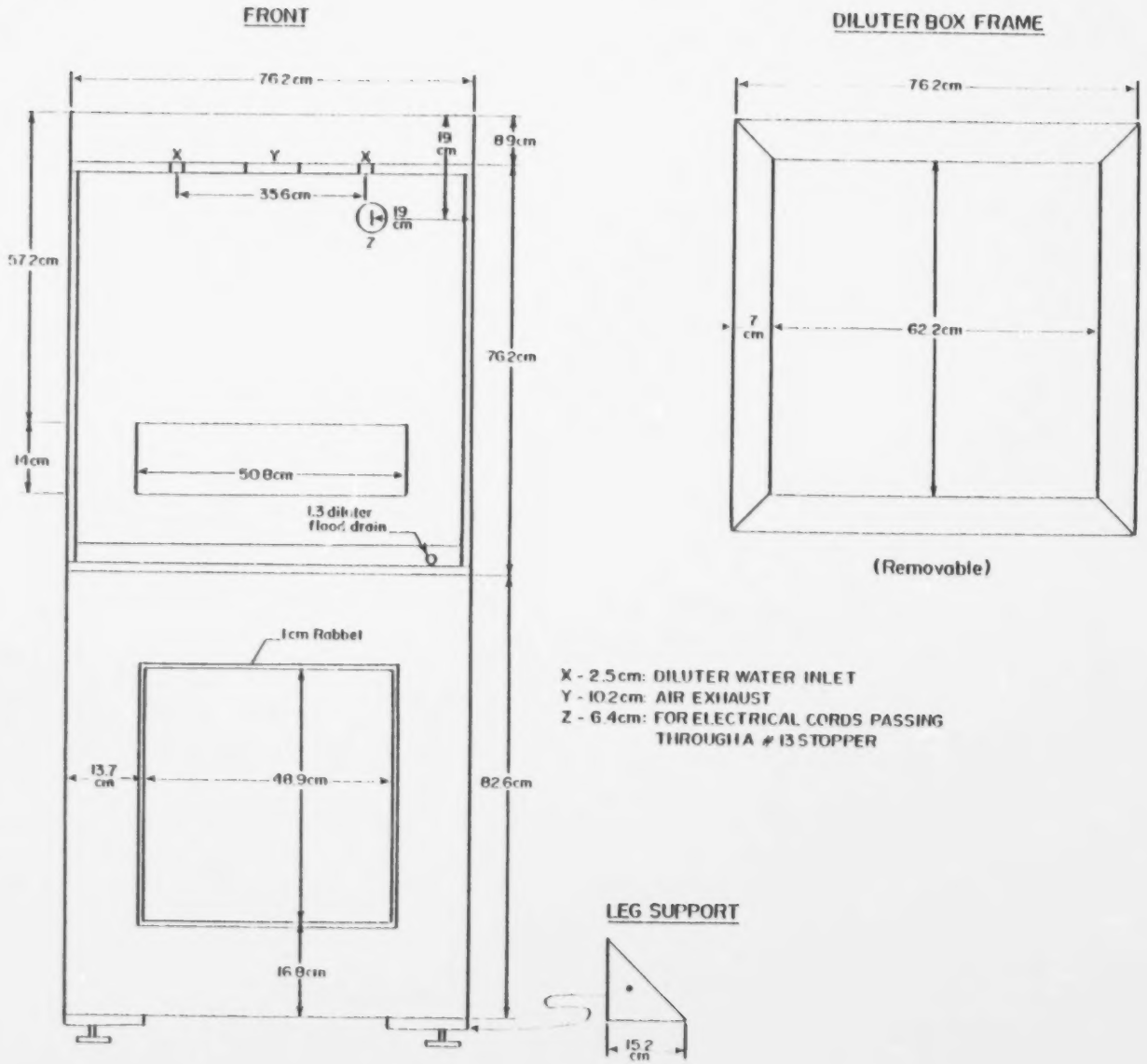

Figure 10. (Continued)

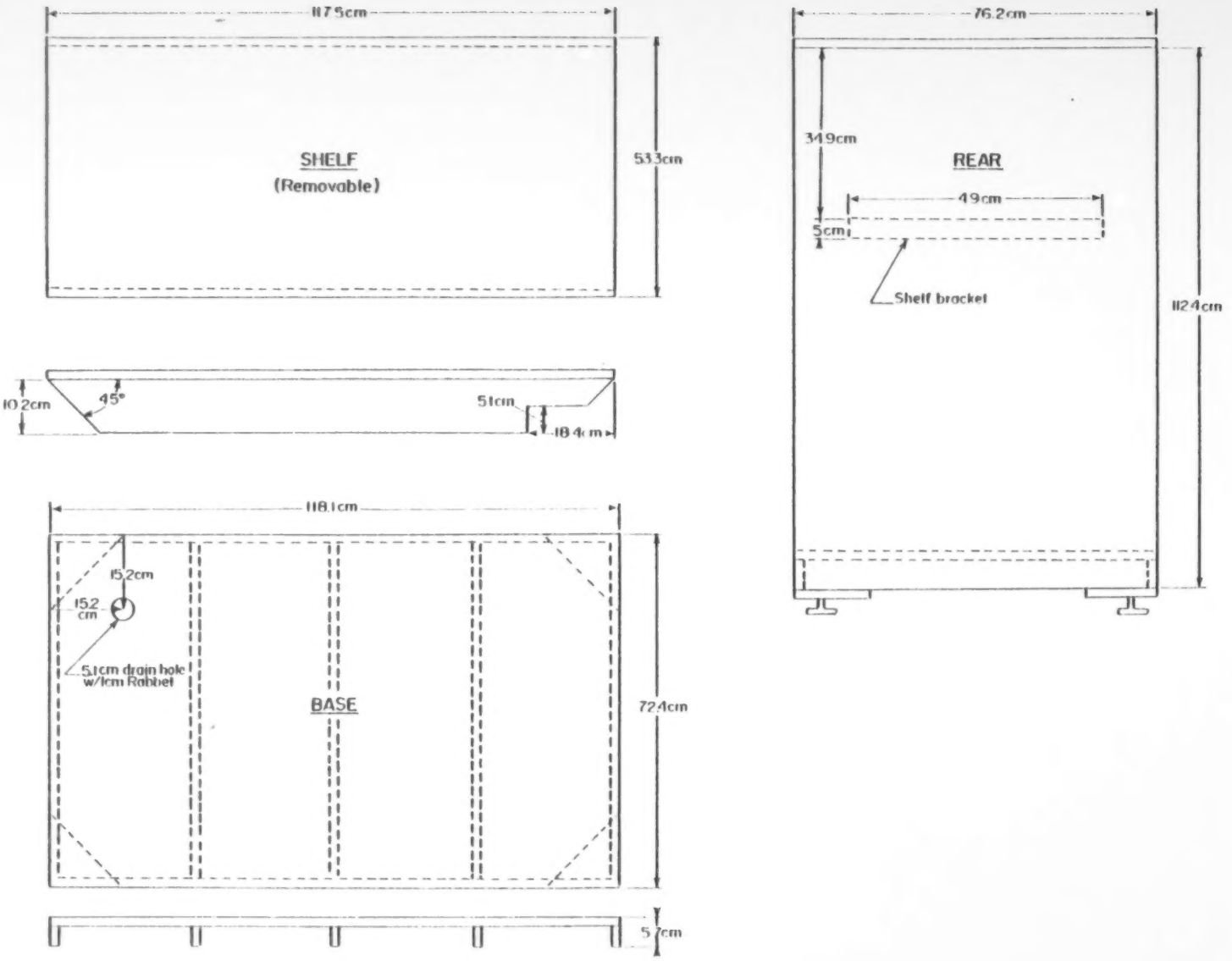

Figure 10. (Continued)

Table 2. Dimensions and Number of 1.9 cm Exterior Plywood Pieces and Other Materials Needed to Construct One Vented Enclosure Shown in Figure 10

Exposure Box[1]:

base - 72.4 x 118.1 cm (1)
3.8 x 118.1 cm (2)
3.8 x 68.6 cm (4)
front - 76.2 x 167.6 cm (1)
side - 112.4 x 118.1 cm (2)
rear - 76.2 x 112.4 cm (1)
top - 76.2 x 120 cm (1)
shelf - 53.3 x 117.5 cm (1)
10.2 x 117.5 cm (2)
shelf bracket - 5 x 49 cm (2)

Diluter Box[1]:

sides - 18.4 x 72.4 (2)
top - 18.4 x 76.2 cm (1)
bottom - 18.4 x 76.2 cm (1)
front frame - 7 x 76.2 cm (4)
flood baffle - 5 x 72.4 cm (1)

Sliding 6 mm Plate Glass Doors: (all edges rounded)

sides[2] (upper) - 30.5 x 30.5 cm (6)
right side[2] (lower) - 45.7 x 45.7 cm (2)
top - 25.4 x 91.4 cm (2)
diluter box[2] - 31.8 x 61 cm (2)

6 mm Sheet Plastic for Lower Front and Left Side: 50.8 x 50.8 cm (2)

E-Z Glide® Aluminum Track: Upper channel, 3.66 M; lower channel, 7.32 M

Miscellaneous Equipment: Snap holders for Plexiglass, (8): leg levelers (4); weatherstrip for removable diluter frame and exposure box top, 1.9 cm wide; weatherstrip for exposure box top glass, 1 cm wide; PVC bulkhead for waste water drain (1.3 cm) and base drain (2.5 cm).

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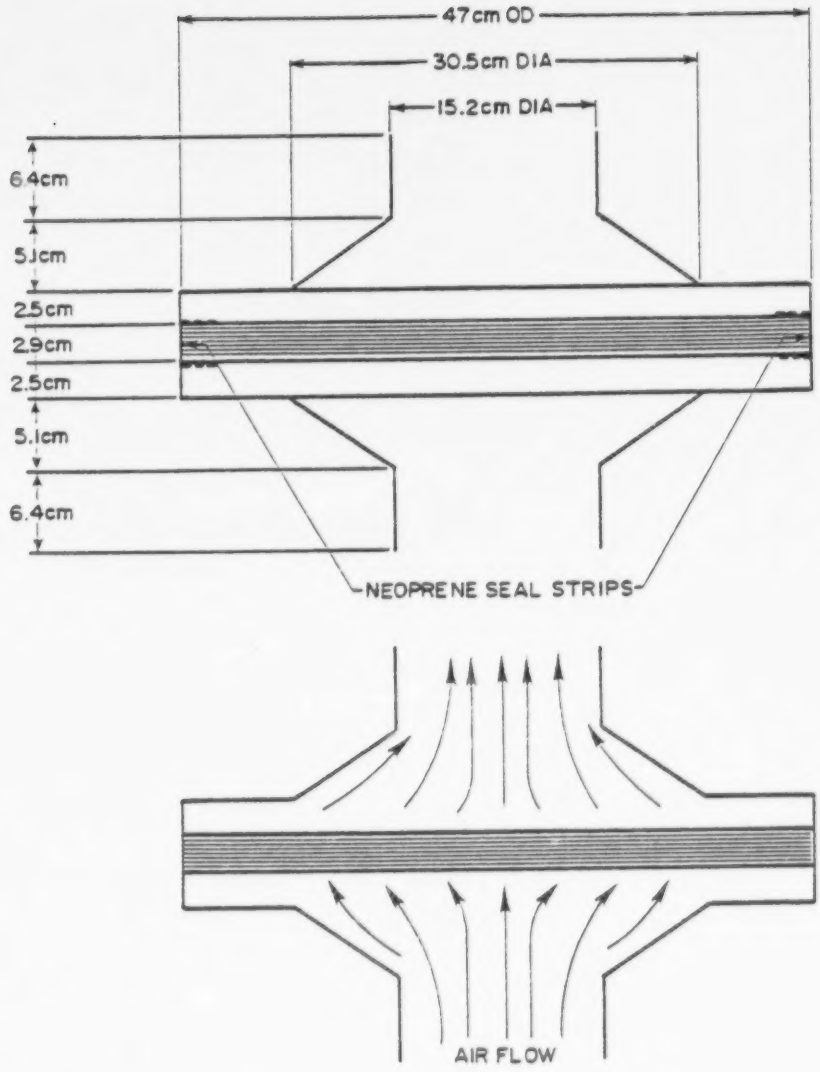
[1] Bottom must be water tight.

[2] Sliding glass doors with finger notches cut in on one side.



MATERIAL: 24 GA GALVANIZED METAL
UNIT: 47 CM x 47 CM
FRONT and REAR OPEN FOR PANEL INSERTION and REMOVAL
SIDES WITH PADDED SEALS FOR AIRTIGHT FIT

Figure 11. Carbon panel adsorber frame used with the vented enclosure for purification of exhaust air.

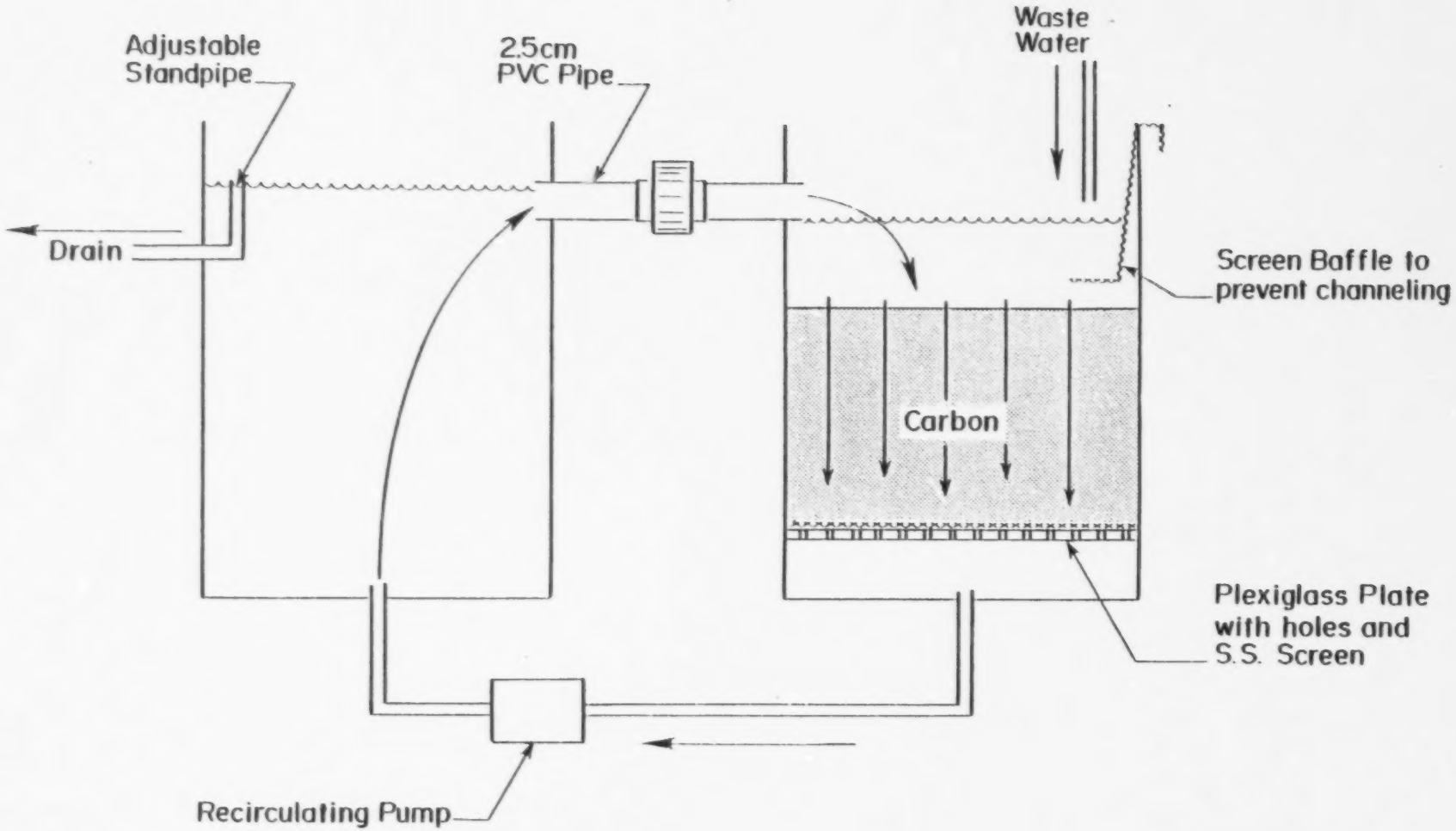


Figure 12. Waste water carbon filter for low-flow exposure systems (0.5 L/min). Two or more units can be used in series to increase filtration. Debris siphoned off exposure chamber bottoms should not be dumped on top of the carbon, but can be filtered out of the siphoned test water with glass wool.

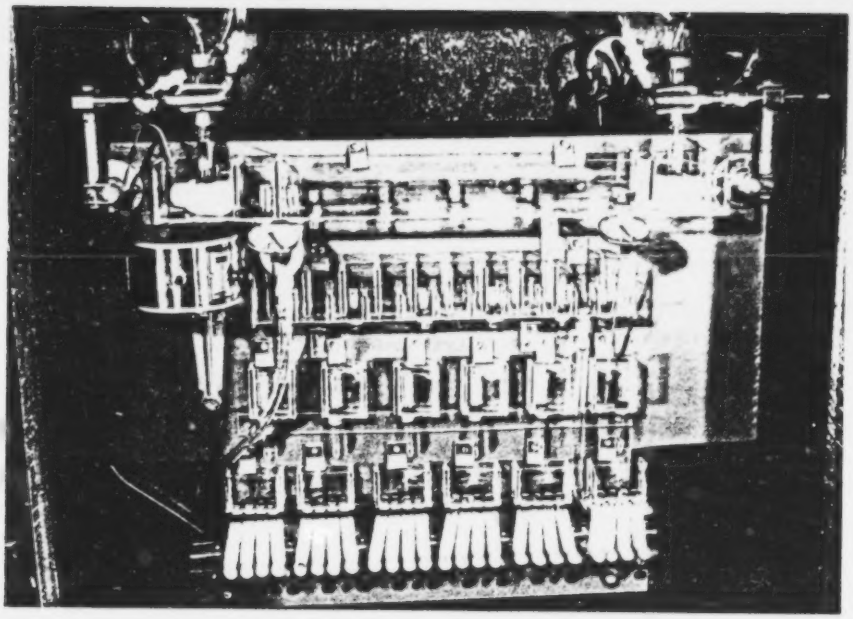


Figure 13. Continuous flow mini-diluter for use with either single chemicals or treated complex effluents.

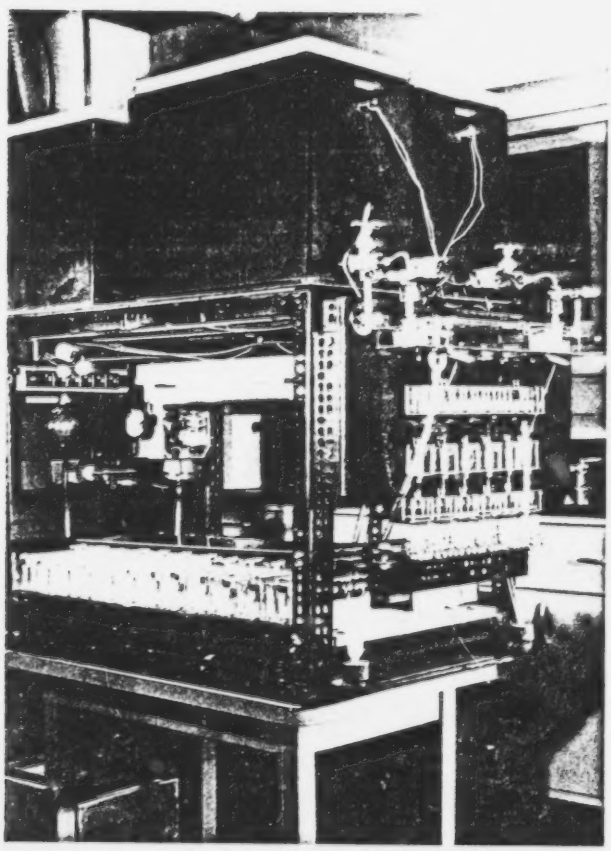


Figure 14. Portable early life stage exposure system

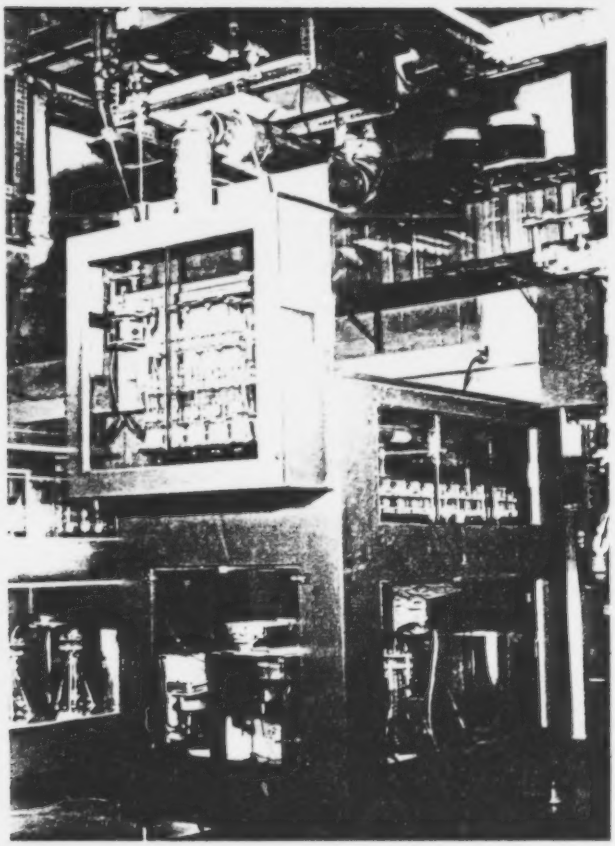


Figure 15. Stationary vented early life stage exposure system.

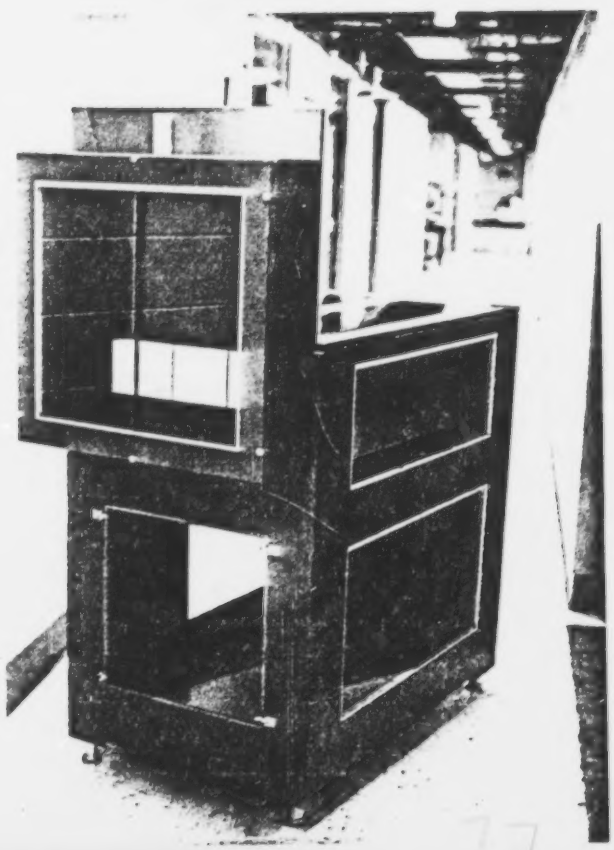
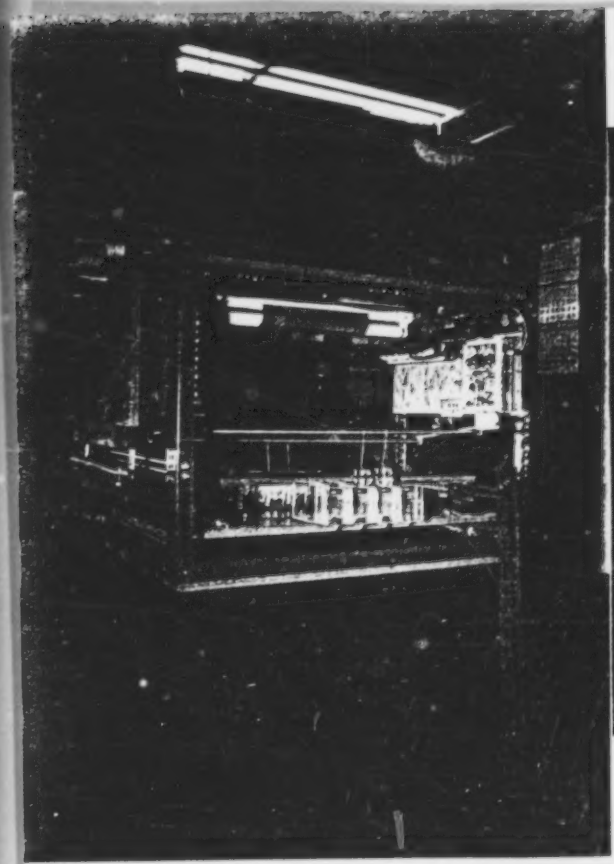


Figure 16. Vented enclosure for testing hazardous volatile chemicals.



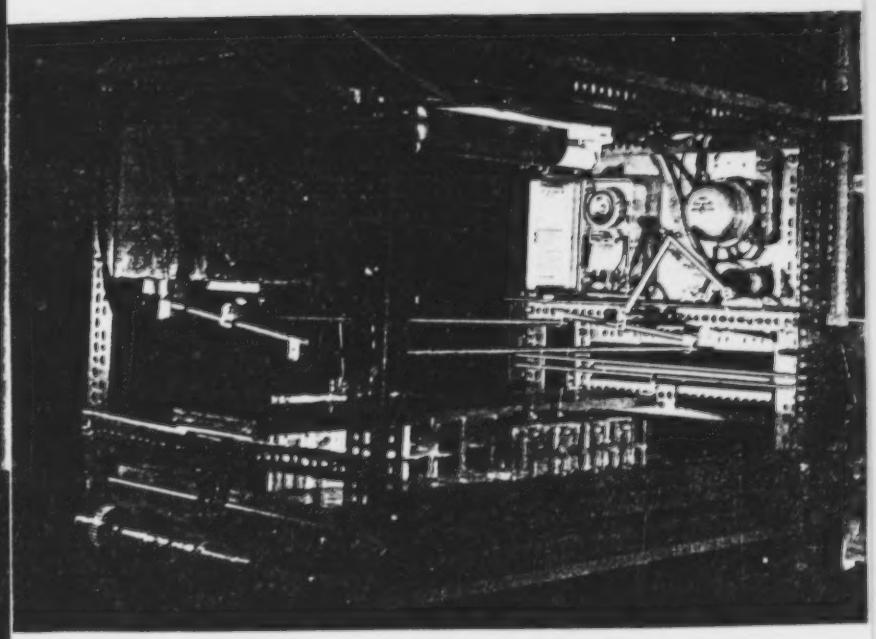
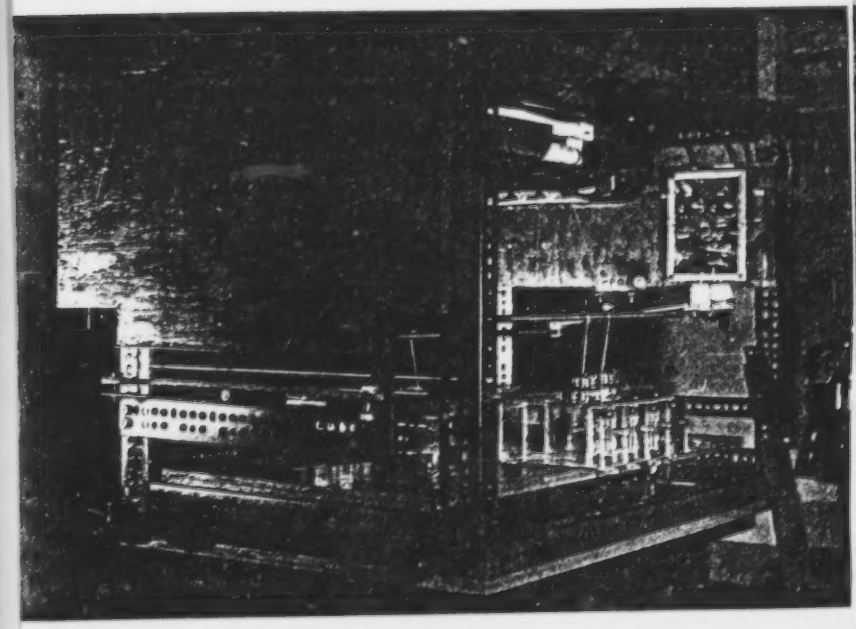


Figure 17-19. Steel support frame (66 x 102 x 74 cm high) and hardware for portable exposure system.



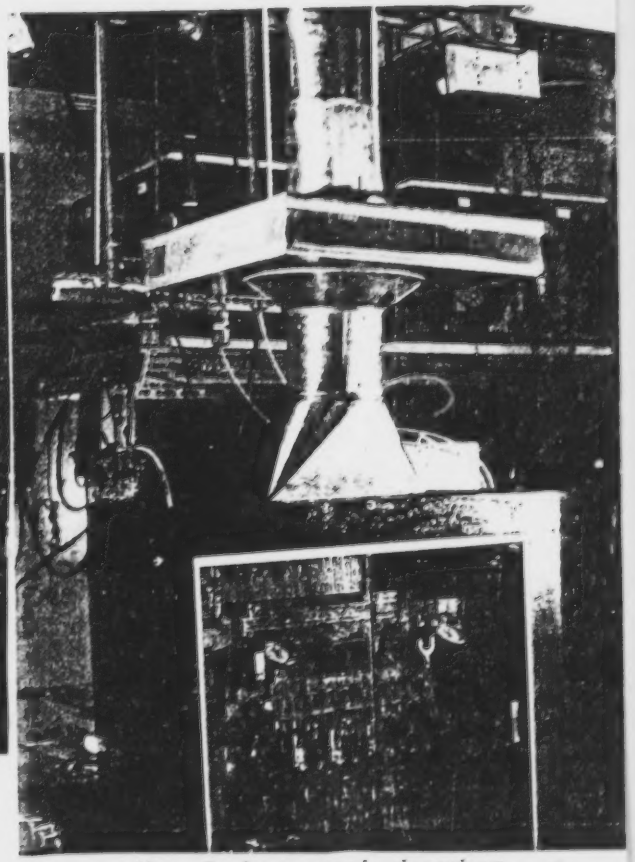


Figure 20. Carbon panel absorber frame attached to a 5.1 x 41 cm sheetmetal adapter for vented enclosure.

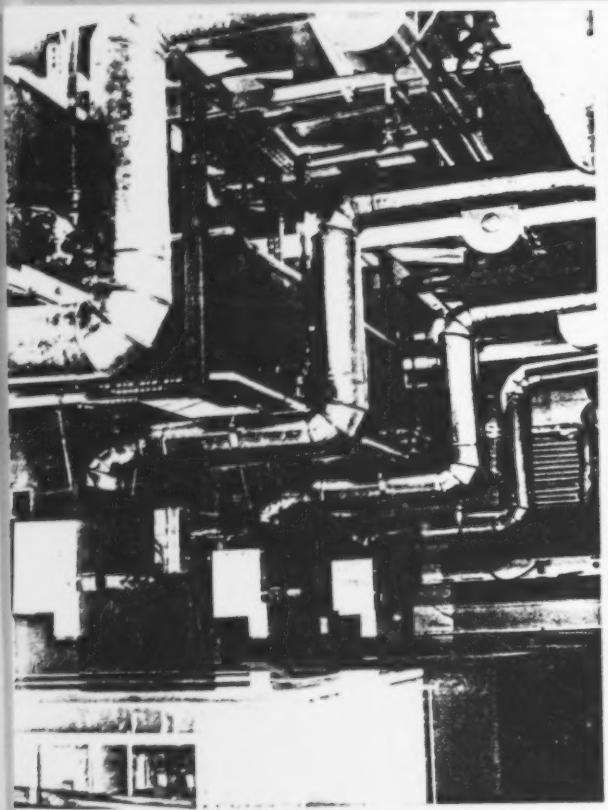


Figure 21. Vent pipes for enclosure systems.

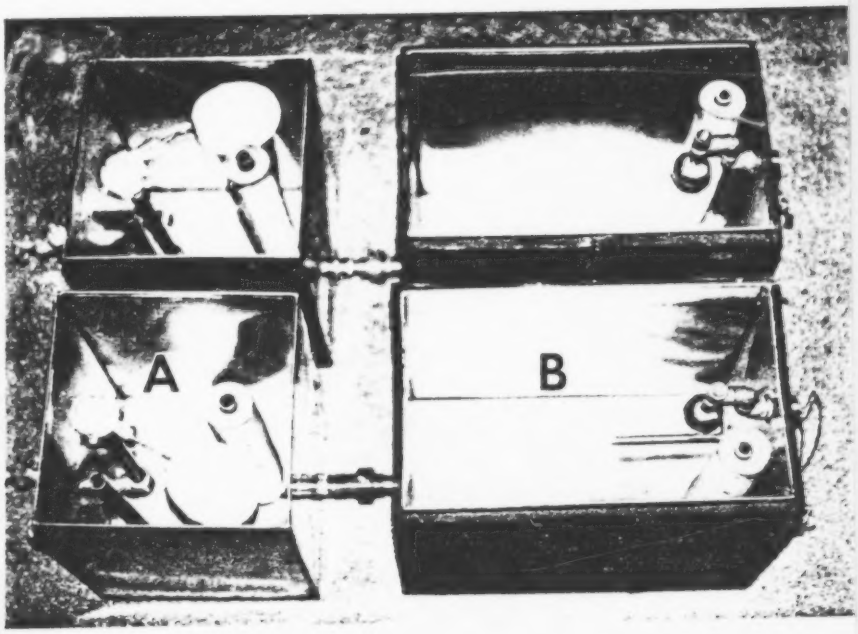
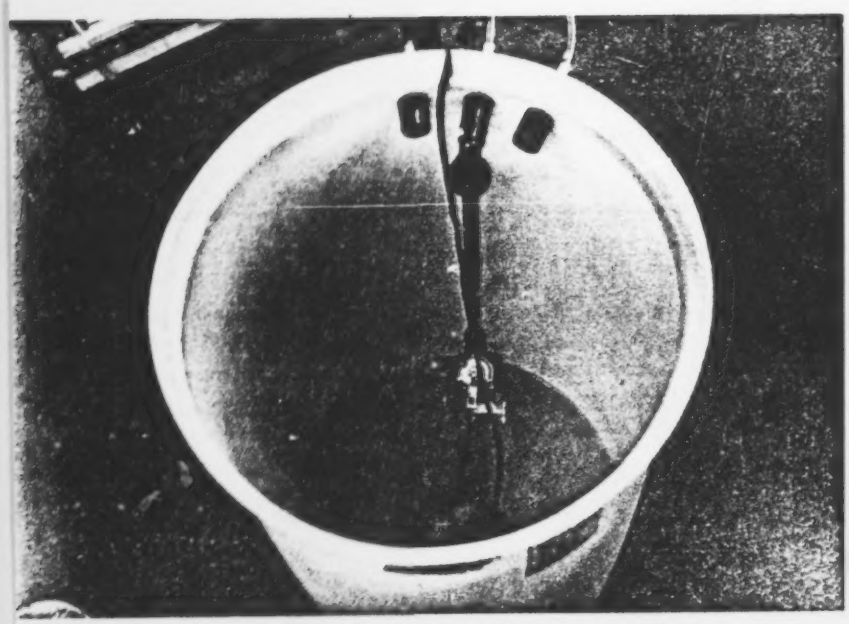


Figure 22. Headboxes for either diluent water or treated complex effluents. Legend (A) headbox with float valve; (B) insulated headbox with immersion heater. Water flows by gravity from A to B through an interconnecting pipe.



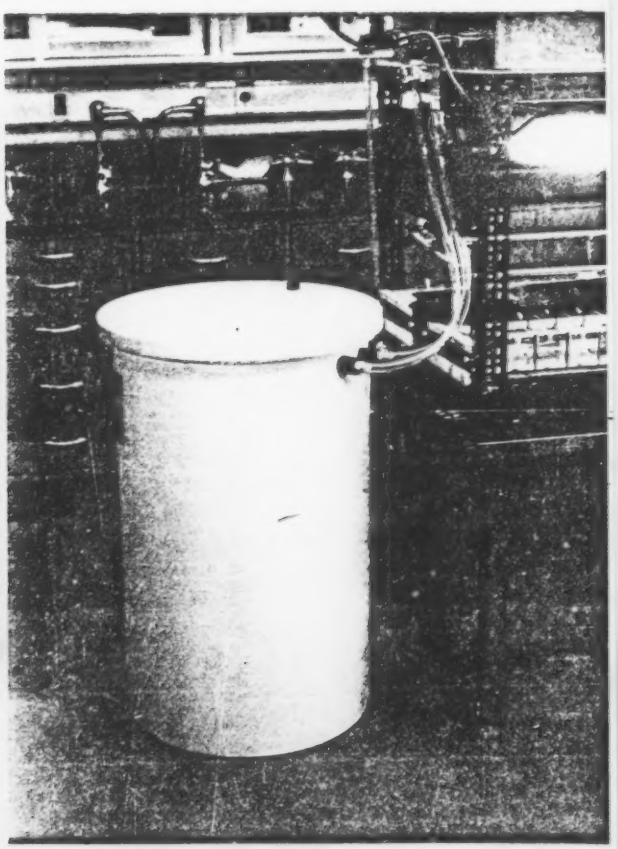


Figure 23 and 24. Fifty five gallon effluent holding drum.

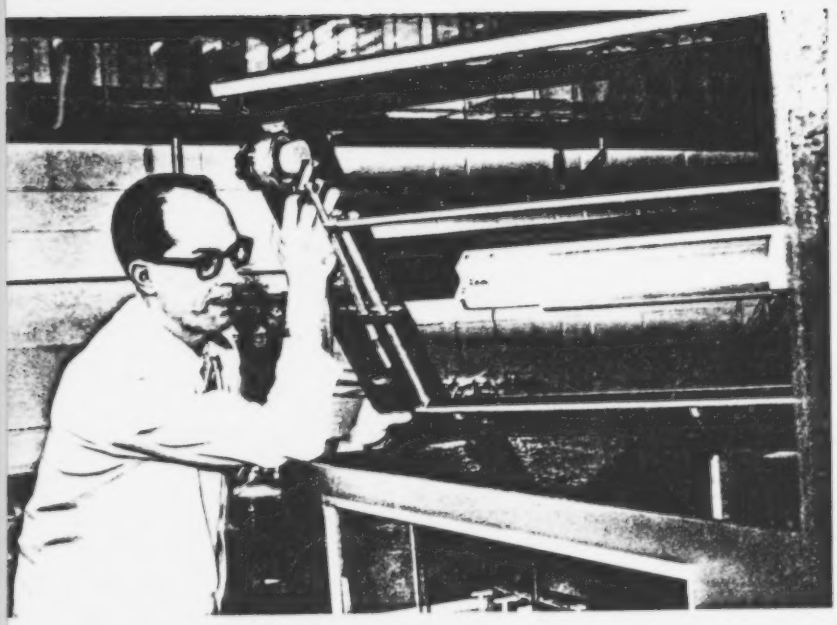


Figure 25. Rocker arm assembly and light attached to removable top of vented enclosure.

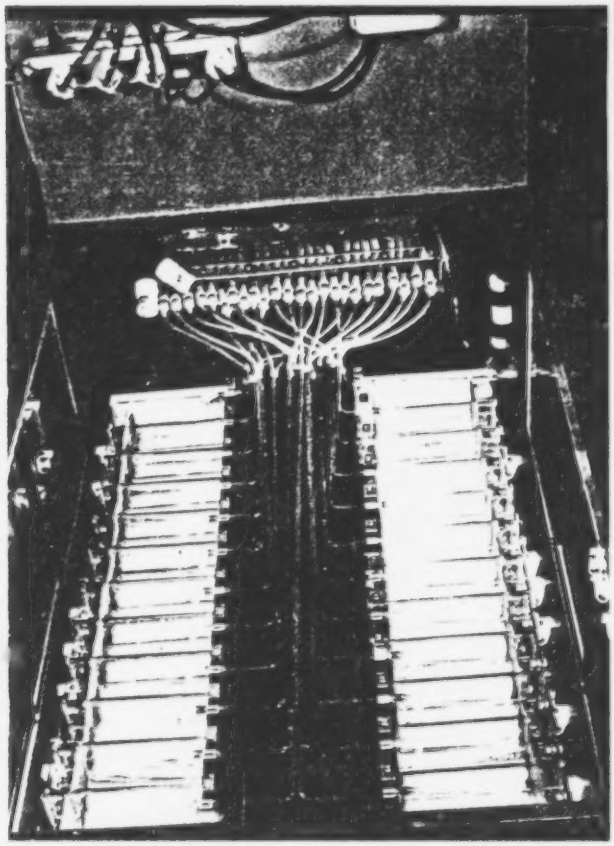
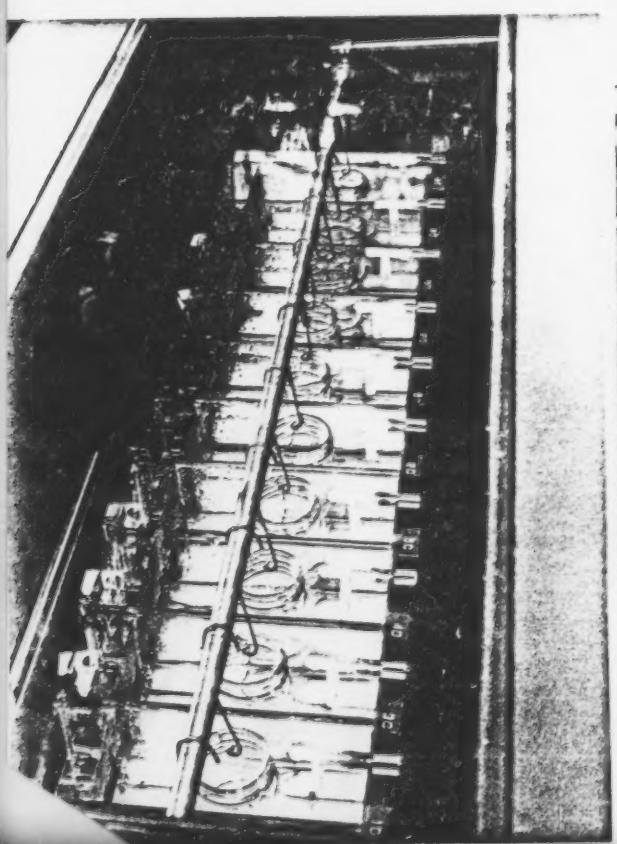


Figure 26. Delivery tubes showing stratified random assignment.



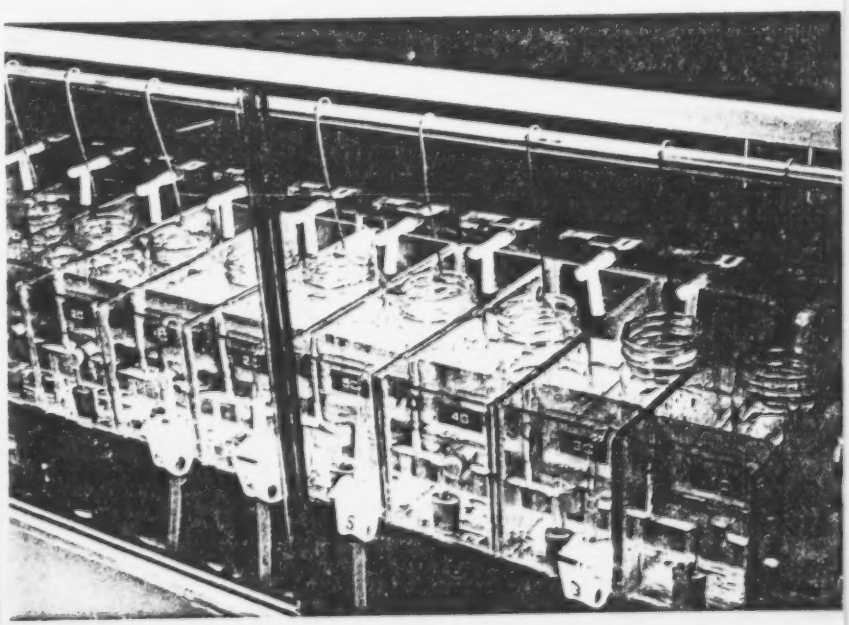


Figure 27 and 28. Exposure chambers with egg cups on rocker arm.

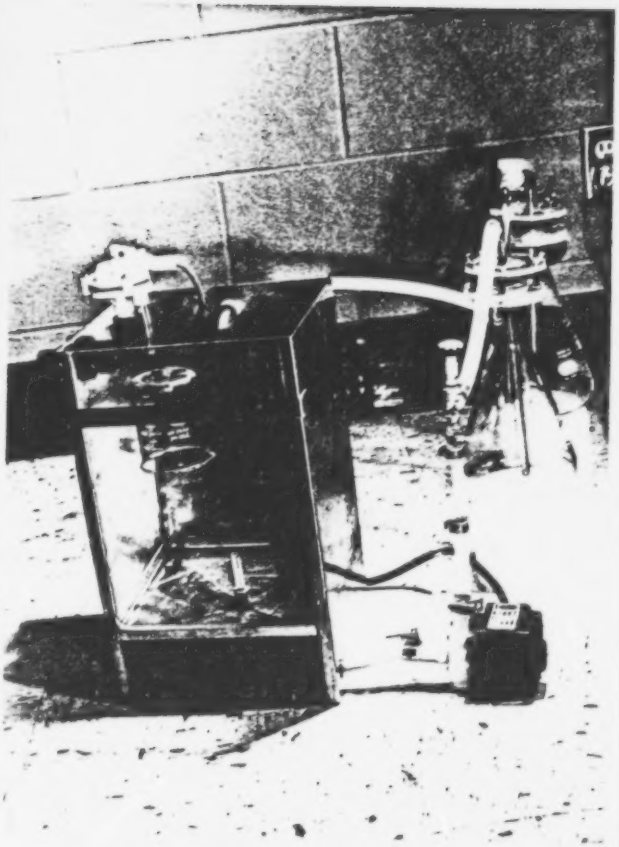


Figure 29 and 30. Saturator for either solid or liquid hydrophobic chemicals showing reservoir with float valve for make-up water, recirculating pump, chemical flask, and liquid chemical transfer flask.

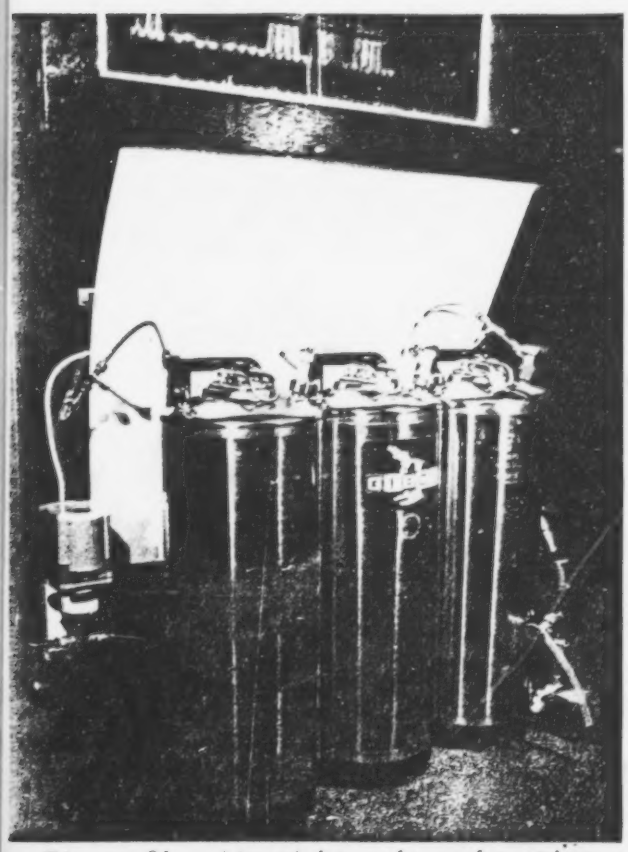


Figure 31. Air tight soda carbonation can saturators can safely be be used outside of enclosure.

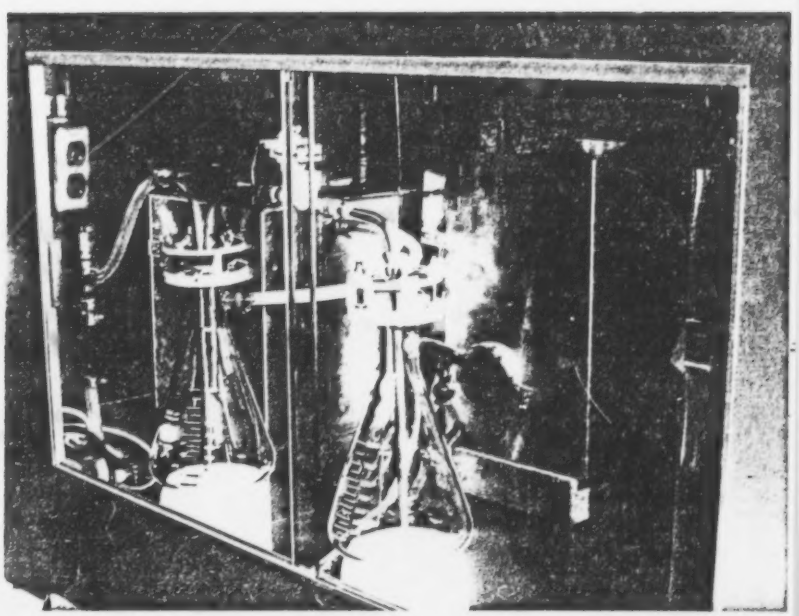


Figure 32. Chemical saturator in lower section in vented enclosure. Chemical flask is inverted for those chemicals lighter than water.

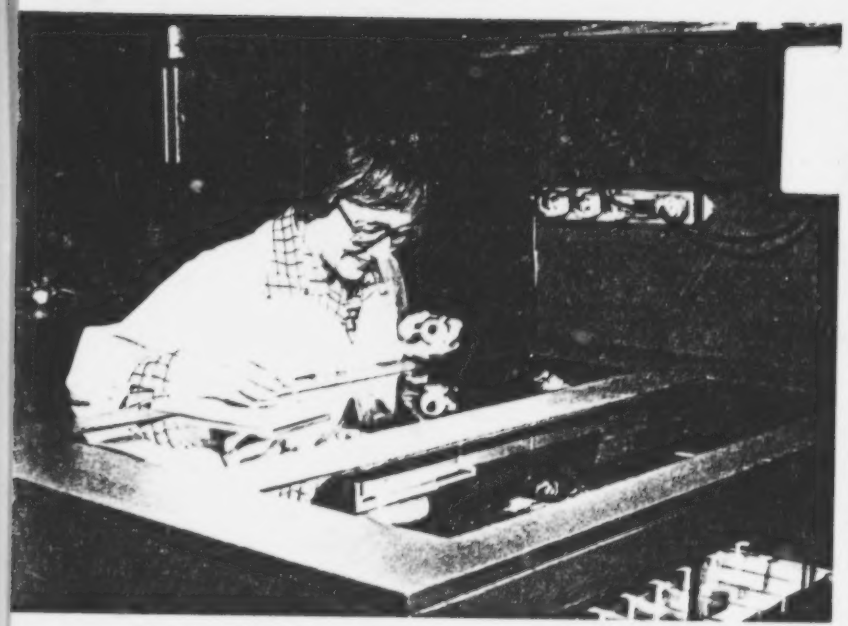


Figure 33-36. Vented enclosure protects the investigator from exposure to toxic fumes.

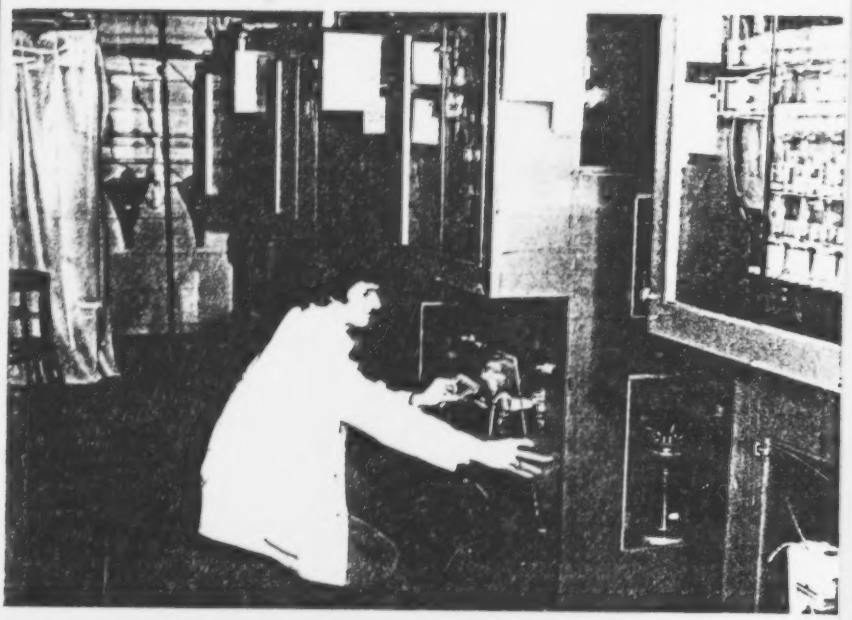


Table 3. Source List For Major Equipment Used With The Mini-Diluter Exposure System

I. Test Water Temperature Control:

A. Syrett-Dawson temperature controller:

(Maximum rating: 500 watt with 8 amp fuse)

R. F. Syrett, and W. F. Dawson. 1975. An inexpensive solid-state temperature controller. Prog. Fish-Cult. 37: 171-172.

B. Heavy duty mercury relay: Model 760

Quick-set thermo-regulator: Model 7501

(Optional temperature controller)

H-B Instrument Co., American & Bristol St., Philadelphia 40, Pa.

C. Stainless steel immersion heater with 1.3 cm pipe threads:

500 watt-RIS-505

750 watt-RIS-755

Volco Co., 831 S. 6th St., Minneapolis, Mn. 55415

II. Vented Exposure Air Exhaust:

A. Exhaust booster fan: Auto-draft inducer model DJ-2

Tjernlund Products Inc., 1620 Terrance Drive, St. Paul, Mn. 55113

B. Carbon panel adsorber: Model PRC, 45.7 x 45.7 x 2.5 cm

Barnebey Cheney, N. Cassady at E. 8th Ave., Columbus, Oh. 43219

III. Light:

A. Light timer: Model 101-G

Tork Time Controls Inc., Mount Vernon, N.Y.

B. Fluorescent light fixture 61 cm: Model 5-120-TS-120-ACLC

Lithonia Lighting, Box A, Conyers, Ga. 30207

IV. Chemical Saturator:

A. Metering pump drive: RP-G50 or 150

Metering pump head: SSY 1 or 2

Fluid Metering Inc., 29 Orchard St., Oyster Bay, N.Y. 11771

B. Combination Open Air - Submersable Pump: Model 1-1C

March Manufacturing Co., 1819 Pickwick Ave., Glenview Ill. 60025

V. Waste Water Filter:

A. Combination Open Air - Submersable Pump: Model 1-1C

March Manufacturing Co., 1819 Pickwick Ave., Glenview, Ill 60025

B. Activated Carbon: 6 x 8 pellets, coconut base

Union Carbide Corp. (Linde Division), P. O. Box 372, 51 Cragwood Rd., South Plainfield, N.J. 07080

VI. Alarm System:

A. Float switch: Model LS-1950

Load-Pak Relay: Model ST-22160

Gems Division, Farmington, Conn. 06032

B. Alarm bell: Model 340

Edwards Co., Inc., Norwalk, Conn.

VII. Effluent Holding Drum:

A. Combination open air - submersible pump: Model 1-1C

March Manufacturing Co., 1819 Pickwick Ave., Glenview, Ill. 60025

B. 208 l polyethylene storage tank with spigot and cover: Number 04032

U.S. Plastic Corp., 1390 Neubrecht Rd., Lima, Oh. 45801

VIII. Effluent or Diluent Water Headbox:

304 stainless steel, 20 gauge with welded seams and 1.3 cm stainless steel couplings welded in place: headbox A - 30.5 cm wide x

30.5 long x 40.6 cm high; headbox B - 30.5 cm wide x 45.7 cm long x 40.6 cm high. A. G. O'Brien or Chester Zimm Sheet Metal Works, Duluth, Mn.

IX. Egg Cup Rocker Arm Assembly (as shown in Fig. 17-19 and 25):

A. 2 RPM induction geared motor (RMS Motor Corp.)

Blan Electronics Corp., 52 Warren St., New York, N.Y. 10007

B. Aluminum Flexaframe rods and fittings

Fisher Scientific, 1600 W. GlenLake Ave., Itasca, Ill. 60143

X. Magnetic Stirrer for Diluter:

Stir-mate: Model 214-957

Curtis Matheson Scientific Inc.

XI. Inert Flexible Tubing for Diluter System and Saturator:

Bev-A-Line: 5 mm ID, 1 mm wall thickness

: 8 mm ID, 2 mm wall thickness

Thermoplastic Scientifics Inc., 57 Stirling Rd., Warren, N.J. 07060

XII. Stainless Steel Screen: (20 mesh, .016 wire; 40 mesh, .010 wire)

W. S. Tyler Co. Inc., 8200 Tyler Blvd., Mentor, Oh. 44060

XIII. Diluter Float Valves:

A. Aluminum Flexaframe rods and fittings

Fisher Scientific, 1600 W. GlenLake Ave., Itasca, Ill. 60143

B. Tapered micro stopper

Scientific Products, 1210 Leon Place, Evanston, Ill. 60201

C. Nalgene® 30 mL bottle

Scientific Products, 1210 Leon Place, Evanston, Ill. 60201

Aquatic Toxicity Tests to Characterize the Hazard of Volatile Organic Chemicals in Water: A Toxicity Data Summary -- Part II

Final Data Summary Report: Phase I, Microsomal Metabolism and Binding of Carbon Tetrachloride, Chloroform, 1,1,2-Trichloroethane, 1,1,2-Trichloroethylene and Monochlorobenzene by Microsomal Fractions of Rainbow Trout (*Salmo gairdneri*) and Water Flea (*Daphnia magna*)

INTRODUCTION

Halogenated hydrocarbons are among the most widely utilized industrial chemicals. They are used as solvents, degreasers and intermediates in chemical synthesis. Because of their desirable chemical and physical properties and reasonable cost, a large volume of chlorinated aliphatic and benzene compounds are used for manufacturing a variety of products. Some of these haloalkanes are known central nervous system depressants, hepatotoxins, nephrotoxins and proven carcinogens (Anderson and Scott, 1981). Many haloalkanes are listed as priority pollutants by the Environmental Protection Agency.

The present study was designed to assess the *in vitro* comparative metabolism and protein binding of carbon tetrachloride, chloroform, 1,1,2-trichloroethylene, 1,1,2-trichloroethane and monochlorobenzene by microsomal fractions of rainbow trout (*Salmo gairdneri*) liver and by post-mitochrondrial supernatant (PMS) fractions of the water flea (*Daphnia magna*). Hepatotoxic and nephrotoxic effects of some of these compounds in mammals have been extensively studied and reviewed in recent years (Plaa, 1977; Ahmed *et al.*, 1980; Tsyrlov and Lyakhovich, 1975; Rechnagel, 1967) but very little information is available concerning the metabolic disposition or protein binding in fish species and aquatic food chain organisms, such as *Daphnia* sp. Previous studies have shown that carbon tetrachloride and other chlorinated benzenes have hepatotoxic effects on fish liver (Pfeifer and Weber, 1979; Gingerich and Weber, 1979; Gingerich *et al.*, 1978; Statham *et al.*, 1978). Recent evidence suggests that carbon tetrachloride, chloroform and other chlorinated alkanes are converted to toxic metabolites by the microsomal mixed function oxidase system (Docks and Krishna, 1976; Watanabe *et al.*, 1978) in mammalian liver. Therefore, in this investiga-

tion rainbow trout liver microsomes and Daphnia PMS were used to determine the formation of water soluble metabolites and protein bindings of these haloalkanes.

METHODS AND MATERIALS

Chemicals: Uniformly ^{14}C labelled 1,1,2-trichloroethylene and 1,1,2-trichloroethane were purchased from California Bionuclear Corporation, 7654 San Fernando Road, Sun Valley, California 91352. Uniformly ^{14}C labelled 1-chlorobenzene, chloroform and carbon tetrachloride were purchased from New England Nuclear Corporation, 549 Albany Street, Boston, Massachusetts 02118. Purity of these compounds ranged between 98-99 percent as determined by gas-liquid chromatography. NADPH, NADP, glucose-6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase from torula yeast, and cytochrome C were purchased from Sigma Chemical Company, St. Louis, Missouri.

Tissue Preparations: Livers were dissected from 3-5 rainbow trout (350-400 g), weighed, and cut into thin slices in cold (4 C) 0.15 M KCl solution. Liver slices were washed several times with KCl (0.15 M) to remove hemoglobin and red blood cells, transferred to 0.1 M pH 7.5 sodium phosphate buffer and homogenized by 6-8 passes of a teflon pestle in a Potter-Elvehjem glass homogenizer. Homogenates of 30-40% liver by weight in phosphate buffer were centrifuged twice at 10,000 g for 15 min in a Beckman L5-50 ultra-centrifuge with a 50 Tirotor to remove nuclear and mitochondrial fractions, which were discarded. The 10,000 g supernatant was centrifuged at 105,000 g for 60 min using a T150 rotor. The supernatant was discarded and the pellet was stored at -20 C until used.

Adult Daphnia (approximately 21 days old) were reared in the laboratory from U.S. EPA Environmental Research Laboratory-Duluth brood stock. They were collected on Whatman #1 filter paper, dried, weighed, and 0.5 - 2.5 g of Daphnia homogenized with a teflon pestle homogenizer. The homogenate was filtered through loose glass wool to remove chitinous materials, and centrifuged twice at 10,000 g to remove nuclear and mitochondrial fractions. The PMS was then frozen at -20 C until used for in vitro metabolic studies.

Protein Determination: Protein determinations were made for Daphnia PMS and rainbow trout liver microsomes according to the method described by Lowry et al. (1951). This enabled known concentrations of protein to be used in the reaction mixture for metabolic studies.

In Vitro Metabolism Studies: Due to the highly volatile nature of carbon tetrachloride, chloroform, chlorobenzene, 1,1,2-trichloroethane and 1,1,2-trichloroethane, an incubation system was designed to study their binding to microsomal protein and their metabolism. This enclosed system consisted of an erlenmeyer flask (125 ml) which was fitted with a glass column (5 mm i.d.) containing two glass wool plugs with approximately 5 cm of silica gel between them to trap the parent compounds being volatilized from the reaction mixture. Another glass column connected the erlenmeyer flask to a CO_2 absorbing system containing a solution of Carbosorb II.® The reaction mixture in the erlenmeyer flask contained an NADPH-generating system (consisting of 3 μM Glucose-6-phosphate, 1 unit[1/] Glucose-6-phosphate dehydrogenase, and 1 μM $MgCl_2$), 8 mg microsomal protein from rainbow trout liver or 4 mg PMS protein from Daphnia, in 0.07 M sodium phosphate buffer (pH 7.5) and 0.1 ml of test compound with known amount of radioactivity made to a final volume of 5 ml. The reaction

1/ One unit will oxidize 1.0 μM of D-glucose 6-phosphate to 6 phospho-D-gluconate per minute in the presence of NADP at pH 7.4 at 25 C.

mixture was incubated in a shaking water bath at a temperature of 24 ± 2 C. The reaction was initiated by addition of radioactive compound (0.1 ml) and was continued for 0, 15, 30, 45, 60 and 120 min with rainbow trout liver microsomes or 0, 15 and 30 min with PMS from Daphnia. The reaction was terminated at various time intervals by addition of 1 ml of 3 M trichloroacetic acid (TCA) solution. The reaction mixture was then extracted thrice with 10 ml of hexane and the extracts pooled. Total percent recovery was determined by summation of the radioactivity in the various fractions as compared to the known amount of radioactivity added initially. Recoveries ranged from 91.4 to 29.2% with recovery efficiency decreasing with time. The loss likely occurred by escape of ^{14}C through the silica gel column and the air space within the reaction vessel becoming saturated with parent compound or metabolites.

Aliquots of the hexane extract (representing parent compound) were transferred to scintillation cocktail (10 ml Permaflour III®, 33 ml Triton X-100, 57 ml scintillized toluene) and ^{14}C radioactivity was counted with a Packard Model 3375 liquid scintillation spectrometer for 5 min. Background and quench corrections were made for all counts. The aqueous phase (representing water soluble metabolites) was then centrifuged at 2200 g with International Model PR-2 centrifuge for 20 minutes and the radioactivity determined in the supernatant and the floating protein pellet. This method distinguished between protein bound and free radioactivity present in the aqueous phase which was unextractable in hexane. The silica gel was extracted with 30 ml of hexane to determine the amount of radioactivity volatilized from the reaction mixture. The carbon dioxide absorbing solution was counted to determine radioactivity evolved as

CO_2 during the metabolic reaction or volatilized as parent compound. The analysis was performed three times with three batches of tissue preparation.

Enzyme Activity: Cytochrome P-450 and chtochrome b_5, were determined by difference spectroscopy with a Beckman DB-G spectrophotometer according to the methods of Omura and Sato (1964). NADPH-cytochrome c-reductase activity was determined by the method described by Williams and Kamin (1962). Aniline hydroxylase activity was determined by measuring the amount of p-aminophenol produced during a 30-min incubation of the liver microsomes or the PMS with aniline hydrochloride at 24 C. The reaction mixture contained an NADPH-generating system as described previously 1 μM mole of aniline hydrochloride and 5 mg microsomal protein. The reaction was stopped by addition of 0.5 ml 3 M TCA. After centrifugation of the reaction mixture at 2200 g for 20 min., a 1 ml aliquot of the reaction mixture was made basic with 0.5 ml of 10% Na_2CO_3 and a blue phenol indophenol complex was formed by addition of 1 ml of 2% phenol in 0.2 N NaOH. Absorbance was measured using a Beckman DB-G spectrophotometer at 630 nm.

RESULTS AND DISCUSSION

Measurements were made showing the distribution of radioactivity after ^{14}C-labeled carbon tetrachloride incubation with trout liver microsomes and Daphnia PMS tissue fractions It was also found that most of these compounds were readily volatilized from the reaction mixture in spite of a silica gel trap (Figure 1). This resulted in lower recoveries of the compound at the termination of chemical reaction. The results (Table 1) indicate that parent carbon tetrachloride could be extracted with hexane after incubation with trout liver microsomes or Daphnia PMS for various time intervals. However, the radioactivity in the aqueous phase and the CO_2 traps increased with concomitant decrease of the hexane extracted radiocarbon. The data also indicate that carbon tetrachloride binds slowly with the microsomal protein fractions of the

trout liver and Daphnia PMS. Formation of the aqueous metabolites and the protein binding of carbon tetrachloride does not appear to be linear with time of incubation. It is evident from our results that both species are capable of metabolizing this hepatotoxin in vitro via microsomal mixed function oxidases.

Metabolism of ^{14}C-labeled chloroform in fish and Daphnia shows the metabolism of chloroform by microsomal mixed function oxidase system of trout liver and Daphnia. The data indicate (Table 2) a rapid conversion of chloroform to hexane-unextractable water soluble metabolites in trout liver and Daphnia PMS. Approximately 40% of 80 to 90% radioactivity was found in the aqueous phase and 53% was extracted in hexane within 1 min of its incubation with trout liver microsomes. Similarly, the aqueous phase from Daphnia had more than 50% of the radioactivity in the aqueous phase as compared to about 40% in the hexane extract. The radioactivity in the aqueous phase increased to 70% in the case of Daphnia, while about 45% was found in trout. Measurable radioactivity was also found in the carbon dioxide traps of both animal species. Trout liver microsomes showed increased protein binding with incubation time. However, Daphnia showed little change in protein bound radioactivity with incubation time.

Most (87-94%) of the radioactivity spiked in the microsomal tissue preparation with ^{14}C chlorobenzene was extractable in hexane even after 120 min of incubation time with trout liver microsomes and 60 min with Daphnia PMS. The aqueous phase of the reaction mixture, in both species, showed small percentages (0.6 - 2.3) water soluble products of metabolism. Higher

amounts of protein bound radioactivity were found with trout liver than with Daphnia tissue preparations.

Trout appear to show higher metabolic activity than Daphnia for 1,1,2-trichloroethylene and 1,1,2-trichloroethane (Tables 4 and 5). More polar metabolites of 1,1,2-trichloroethylene and 1,1,2-trichloroethane were formed by trout liver microsomes than Daphnia PMS. Trichloroethane was more readily converted to water soluble products than trichloroethylene in the case of trout. On the other hand, Daphnia converted both of the compounds to aqueous metabolites at similar but at much slower rates than rainbow trout. Both compounds showed protein binding with rainbow trout or Daphnia microsomal mixed function oxidase system in vitro.

Both rainbow trout and Daphnia metabolized chlorofrom most readily and carbon tetrachloride least readily, based upon the percentages of total radioactivity present in the aqueous phase and in the protein bound phase. For the remaining three compounds, the orders were not the same between species. The order for rainbow trout was chloroform > 1,1,2-trichloroethane > 1,1,2-trichloroethylene > chlorobenzene > carbon tetrachloride. The order for Daphnia was chloroform > chlorobenzene > 1,1,2-trichloroethylene > 1,1,2-trichloroethane $\sim$carbon tetrachloride.

Microsomal monooxygenase or mixed function oxidase assays of trout liver and Daphnia PMS fractions were performed. Trout liver microsomes had mean values of 0.28 and 0.19 nanomoles$\cdot mg^{-1}$, of cytochrome P-450 and cytochrome b_5, respectively (Table 6). The level of NADPH cytochrome c reductase activity in trout liver microsomes was 16 nanomoles of cytochrome c reduced$\cdot min^{-1}\cdot mg^{-1}$ protein. This activity appears to be low in rainbow trout as compared to mammalian liver tissue (Table 7). Trout liver microsomes metabolized aniline

at a very slow rate of 0.04 - 0.05 nanomoles^{-}mg^{-1} protein·min^{-1} (Table 6). PMS from adult Daphnia showed a mean value of 42 ± 5.3 nanomoles of cytochrome c reductase activity·min^{-1}·mg^{-1} protein, which was higher than rainbow trout.

Based on the present information, it is apparent that both species possess an active mixed function oxidase system which may play an important role in detoxication of chlorinated hydrocarbons. Perhaps the initial oxidation of these compounds occurs via the mixed function oxidase system in rainbow trout and Daphnia. Toxicity may be related to irreversible protein binding, and lipid peroxidation causing disruption of the endoplasmic membrane. Further metabolic studies of these chemicals should be conducted to determine their interaction with cellular components, and to identify specific metabolites.

Our data indicate (Table 7) that aquatic organisms have measurable but lower mixed function oxidase activity than mammals. However, with similar metabolic systems, the mechanisms leading to toxicity and neoplasia are presumed to be qualitatively similar in all organisms. Therefore, studies with aquatic organisms can be used for important functions. The first is for laboratory screening. Because they are easier, cheaper and faster to rear than mammals, they are economically attractive test organisms. The second is for environmental monitoring. Aquatic organisms are currently being used as sentinels to signal environmental contamination (Black et al., 1980). In summary, both laboratory and field studies using aquatic organisms are recommended for programs in comparative pharmacological testing, short-term screening and environmental monitoring.

TABLE 1. Distribution (% ± standard deviation) of ^{14}C after incubation with ^{14}C carbon tetrachloride for various time intervals with microsomal fractions of rainbow trout (Salmo gairdneri) liver and post-mitochrondrial supernatant of Daphnia magna. (Values are the means of three separately prepared tissue fractions.)

Rainbow Trout

Time (Min)	Hexane[a] Extracted	Aqueous or[b] Unextracted in Hexane	CO_2[c] Trap	Protein[b] Bound	Total %[d] Recovery
0	91.0 ± 5.8	0.56 ± 0.15	0.012 ± 0.016	0.26 ± 0.096	61.8 ± 11.9
15	92.6 ± 4.2	0.69 ± 0.13	0.15 ± 0.132	0.35 ± 0.10	52.7 ± 8.9
30	88.1 ± 3.0	0.73 ± 0.15	0.097 ± 0.095	0.37 ± 0.15	47.9 ± 16.8
45	90.1 ± 0.75	0.89 ± 0.046	0.15 ± 0.15	0.53 ± 0.13	45.6 ± 7.4
60	87.1 ± 1.8	1.2 ± 0.28	0.25 ± 0.21	0.61 ± 0.30	37.5 ± 8.3
120	84.7 ± 5.9	1.1 ± 0.16	0.18 ± 0.20	0.60 ± 0.15	40.0 ± 4.7

Daphnia

0	95.9 ± 1.8	0.72 ± 0.43	0.09 ± 0.02	0.061 ± 0.03	55.8 ± 16.2
15	91.4 ± 1.3	0.99 ± 0.38	0.15 ± 0.14	0.074 ± 0.03	45.9 ± 9.3
30	93.5 ± 2.3	0.91 ± 0.24	0.06 ± 0.08	0.12 ± 0.10	47.1 ± 4.7
45	89.1 ± 1.2	1.3 ± 0.21	0.38 ± 0.13	0.1 ± 0.06	38.3 ± 3.6
60	87.5 ± 0.92	1.5 ± 0.56	0.13 ± 0.18	0.09 ± 0.10	37.5 ± 2.9

[a]Percent of total added dpm in 0·1 ml solution which could be recovered in hexane after the extraction of reaction mixture.

[b]Percent dpm in aqueous fraction (soluble and protein pellet) relative to dpm extractable in hexane.

[c]Percent radioactivity trapped in Carbosorb II[R] relative to dpm extractable in hexane.

[d]Total percent recovery is based on the dpms recoverable in all fractions including the silica gel trap divided by the total added dpm in the reaction mixture.

TABLE 2. Distribution (% ± standard deviation) of ^{14}C after incubation with ^{14}C chloroform for various time intervals with microsomal fractions of rainbow trout (Salmo gairdneri) liver and post-mitochrondrial supernatant of Daphnia magna. (Values are the means of three separately prepared tissue fractions.)

Rainbow Trout

Time (Min)	Hexane [a] Extracted	Aqueous or [b] Unextracted in Hexane	CO_2 [c] Trap	Protein [b] Bound	Total % [d] Recovery
0	53.2 ± 25.3	38.6 ± 25.8	0.25 ± 0.32	1.1 ± 0.61	87.9 ± 25.3
15	45.9 ± 27.2	43.4 ± 27.5	0.09 ± 0.04	4.2 ± 2.6	86.8 ± 14.0
30	44.3 ± 20.7	44.3 ± 28.7	0.19 ± 0.23	3.5 ± 3.2	91.4 ± 15.7
45	46.3 ± 25.9	44.8 ± 26.8	0.18 ± 0.29	4.6 ± 1.7	83.6 ± 12.5
60	42.1 ± 27.7	44.8 ± 26.3	1.2 ± 1.7	4.6 ± 2.0	81.9 ± 14.9
120	45.3 ± 16.7	42.4 ± 17.5	0.73 ± 1.0	5.9 ± 4.2	83.7 ± 11.6

Daphnia

0	39.7 ± 23.4	54.0 ± 25.9	2.4 ± 3.8	1.6 ± 2.4	83.0 ± 14.1
15	40.3 ± 20.7	53.0 ± 22.8	2.3 ± 3.5	1.5 ± 2.5	77.0 ± 14.8
30	37.8 ± 22.8	56.1 ± 23.9	1.7 ± 2.1	1.3 ± 1.9	85.8 ± 11.5
45	25.4 ± 7.3	70.1 ± 6.5	0.52 ± 0.54	1.5 ± 2.0	73.3 ± 28.8
60	26.2 ± 15.4	69.3 ± 16.5	0.11 ± 0.12	1.5 ± 1.5	74.9 ± 14.2

[a] Percent of total added dpm in 0·1 ml solution which could be recovered in hexane after the extraction of reaction mixture.

[b] Percent dpm in aqueous fraction (soluble and protein pellet) relative to dpm extractable in hexane.

[c] Percent radioactivity trapped in Carbosorb II^R relative to dpm extractable in hexane.

[d] Total percent recovery is based on the dpms recoverable in all fractions including the silica gel trap divided by the total added dpm in the reaction mixture.

TABLE 3. Distribution (% ± standard deviation) of ^{14}C after incubation with ^{14}C chlorobenzene for various time intervals with microsomal fractions of rainbow trout (Salmo gairdneri) liver and post-mitochrondrial supernatant of Daphnia magna. (Values are the means of three separately prepared tissue fractions.)

Time (Min)	Hexane Extracted [a]	Aqueous or Unextracted in Hexane [b]	CO_2 Trap [c]	Protein Bound [b]	Total % Recovery [d]
Rainbow Trout					
0	92.2 ± 2.3	0.56 ± 0.06	0.006 ± 0.004	0.4 ± 0.12	75.2 ± 13.4
15	94.4 ± 1.8	0.95 ± 0.15	0.03 ± 0.01	0.60 ± 0.27	58.4 ± 8.3
30	92.5 ± 1.2	1.0 ± 0.28	0.015 ± 0.009	0.79 ± 0.17	50.0 ± 8.3
45	93.4 ± 2.1	1.3 ± 0.21	0.07 ± 0.01	0.8 ± 0.0	42.6 ± 2.5
60	92.3 ± 2.6	1.5 ± 0.17	0.014 ± 0.010	0.56 ± 0.40	39.1 ± 6.6
120	86.9 ± 1.0	1.9 ± 0.20	0.21 ± 0.25	1.2 ± 0.70	29.2 ± 3.5
Daphnia					
0	91.2 ± 4.0	1.2 ± 0.17	0.06 ± 0.004	0.06 ± 0.026	66.4 ± 15.6
15	94.4 ± 2.0	1.7 ± 0.15	0.024 ± 0.006	0.13 ± 0.08	49.1 ± 8.4
30	92.5 ± 2.7	2.1 ± 0.06	0.042 ± 0.03	0.11 ± 0.08	41.3 ± 16.6
45	90.6 ± 2.1	2.1 ± 0.07	0.07 ± 0.014	0.15 ± 0.02	33.3 ± 11.2
60	92.5 ± 2.7	2.3 ± 0.36	0.09 ± 0.010	0.053 ± 0.006	35.7 ± 6.1

[a] Percent of total added dpm in 0.1 ml solution which could be recovered in hexane after the extraction of reaction mixture.

[b] Percent dpm in aqueous fraction (soluble and protein pellet) relative to dpm extractable in hexane.

[c] Percent radioactivity trapped in Carbosorb II^R relative to dpm extractable in hexane.

[d] Total percent recovery is based on the dpms recoverable in all fractions including the silica gel trap divided by the total added dpm in the reaction mixture.

TABLE 4. Distribution (% ± standard deviation) of ^{14}C after incubation with ^{14}C 1,1,2-trichloroethylene for various time invervals with microsomal fractions of rainbow trout (Salmo gairdneri) liver and post-mitochrondrial supernatant of Daphnia magna. (Values are the means of three separately prepared tissue fractions.)

Time (Min)	Hexane Extracted[a]	Aqueous or Unextracted in Hexane[b]	CO_2 Trap[c]	Protein Bound[b]	Total % Recovery[d]
Rainbow Trout					
0	89.0 ± 3.4	1.1 ± 0.11	0.032 ± 0.007	0.09 ± 0.01	63.3 ± 15.9
15	92.2 ± 4.9	1.6 ± 0.06	0.096 ± 0.09	0.08 ± 0.08	54.0 ± 9.2
30	84.5 ± 5.8	6.3 ± 7.5	0.063 ± 0.046	0.4 ± 0.5	49.2 ± 11.9
45	88.3 ± 3.5	2.2 ± 0.15	0.21 ± 0.27	0.16 ± 0.09	39.9 ± 4.4
60	85.4 ± 3.6	1.8 ± 0.35	0.11 ± 0.11	0.14 ± 0.05	46.5 ± 8.2
120	82.8 ± 3.2	2.6 ± 0.80	0.19 ± 0.10	0.31 ± 0.20	32.7 ± 5.9
Daphnia					
0	88.8 ± 2.1	1.03 ± 0.24	0.06 ± 0.01	0.024 ± 0.032	54.6 ± 0.6
15	89.4 ± 1.7	1.56 ± 0.16	0.10 ± 0.04	0.023 ± 0.017	42.4 ± 4.1
30	90.5 ± 3.7	1.8 ± 0.23	0.14 ± 0.09	0.020 ± 0.014	42.7 ± 5.5
45	91.5 ± 5.0	1.9 ± 0.42	0.03 ± 0.014	0.013 ± 0.011	37.4 ± 0.6
60	89.1 ± 1.3	1.95 ± 0.64	0.095 ± 0.007	0.012 ± 0.011	34.5 ± 3.2

[a]Percent of total added dpm in 0.1 ml solution which could be recovered in hexane after the extraction of reaction mixture.

[b]Percent dpm in aqueous fraction (soluble and protein pellet) relative to dpm extractable in hexane.

[c]Percent radioactivity trapped in Carbosorb II^R relative to dpm extractable in hexane.

[d]Total percent recovery is based on the dpms recoverable in all fractions including the silica gel trap divided by the total added dpm in the reaction mixture.

TABLE 5. Distribution (% ± standard deviation) of ^{14}C after incubation with ^{14}C 1,1,2-Trichloroethane for various time intervals with microsomal fractions of rainbow trout (Salmo gairdneri) liver and post-mitochrondrial supernatant of Daphnia magna. (Values are the means of three separately prepared tissue fractions.)

Rainbow Trout

Time (Min)	Hexane Extracted[a]	Aqueous or Unextracted in Hexane[b]	CO_2 Trap[c]	Protein Bound[b]	Total % Recovery[d]
0	81.5 ± 24.7	12.4 ± 20.3	0.22 ± 0.35	0.65 ± 0.91	77.6 ± 21.7
15	79.3 ± 31.3	16.7 ± 27.4	0.049 ± 0.07	1.6 ± 2.5	75.6 ± 18.6
30	76.3 ± 34.1	15.1 ± 24.5	0.67 ± 0.25	1.3 ± 2.1	68.9 ± 16.3
45	77.8 ± 28.7	15.8 ± 25.6	0.18 ± 0.28	1.46 ± 2.3	69.4 ± 13.3
60	77.9 ± 31.7	15.5 ± 24.9	1.1 ± 1.8	1.47 ± 2.2	66.7 ± 11.1
120	76.4 ± 30.9	16.8 ± 26.7	0.70 ± 1.0	1.26 ± 1.5	59.7 ± 9.6

Daphnia

0	96.3 ± 0.56	0.77 ± 0.30	0.005 ± 0.004	0.004 ± 0.004	71.0 ± 14.7
15	97.2 ± 0.96	0.96 ± 0.30	0.007 ± 0.005	0.012 ± 0.008	60.1 ± 19.9
30	96.8 ± 0.75	1.1 ± 0.35	0.036 ± 0.029	0.009 ± 0.001	49.6 ± 7.6
45	97.0 ± 0.78	1.1 ± 0.14	0.015 ± 0.007	0.01 ± 0.014	47.3 ± 13.4
60	97.0 ± 0.0	0.98 ± 0.035	0.02 ± 0.0	0.012 ± 0.011	50.0 ± 18.8

[a]Percent of total added dpm in 0.1 ml solution which could be recovered in hexane after the extraction of reaction mixture.

[b]Percent dpm in aqueous fraction (soluble and protein pellet) relative to dpm extractable in hexane.

[c]Percent radioactivity trapped in Carbosorb II^R relative to dpm extractable in hexane.

[d]Total percent recovery is based on the dpms recoverable in all fractions including the silica gel trap divided by the total added dpm in the reaction mixture.

TABLE 6. Mixed Function Oxidase System of Rainbow Trout (Salmo gairdneri) liver and Daphnia

Enzymes	Rainbow Trout	Daphnia
Cytochrome[a] P-450	0.28 ± 0.1 (4)[d]	N.D.
Cytochrome b_5[a]	0.19 ± 0.05 (4)	N.D.
NADPH Cytochrome[b] c-reductase	15.9 ± 2.2 (8)	42 ± 5.3 (3)
Aniline hydroxylase[c]	0.05 ± 0.01 (3)	-

[a] nanomoles·mg^{-1} microsomal protein ± S.D.

[b] nanomoles of cytochrome c reduced·min^{-1}·mg^{-1}protein ± S.D.

[c] nanomoles of p-aminophenol formed·min^{-1}·mg^{-1}protein ± S.D.

[d] numbers in parentheses are the number of tissue preparations from separate animal batches

TABLE 7. Comparison of mixed function oxidase measurements between mammals and several non-mammalian aquatic organisms

Enzymes[a]	Human	Male Rat[c]	Rainbow Trout[d]	Daphnia[d]	Blue Crab[e]
Cytochrome P-450	0.60 ± 0.10[b]	0.72 ± 0.08	0.28 ± 0.10	ND[f]	0.18 ± 0.08
Cytochrome b_5	0.49 ± 0.06[b]	0.30 ± 0.08	0.19 ± 0.05	ND	-
NADPH Cytochrome c reductase	102.6 ± 14.6[b]	96 ± 20	15.9 ± 2.2	42.0 ± 5.3	5.2 ± 4.8
Aniline hydroxylase	8.7 ± 6.8[c]	22 ± 5	0.05 ± 0.01	-	0.016 ± 0.008

a Activities expressed as in Table 6.

b Ahmad and Black, 1977

c Kato, 1979; Tables 27 and 38.

d This study.

e James et al., 1979; Tables 3 and 4.

f Not detectable.

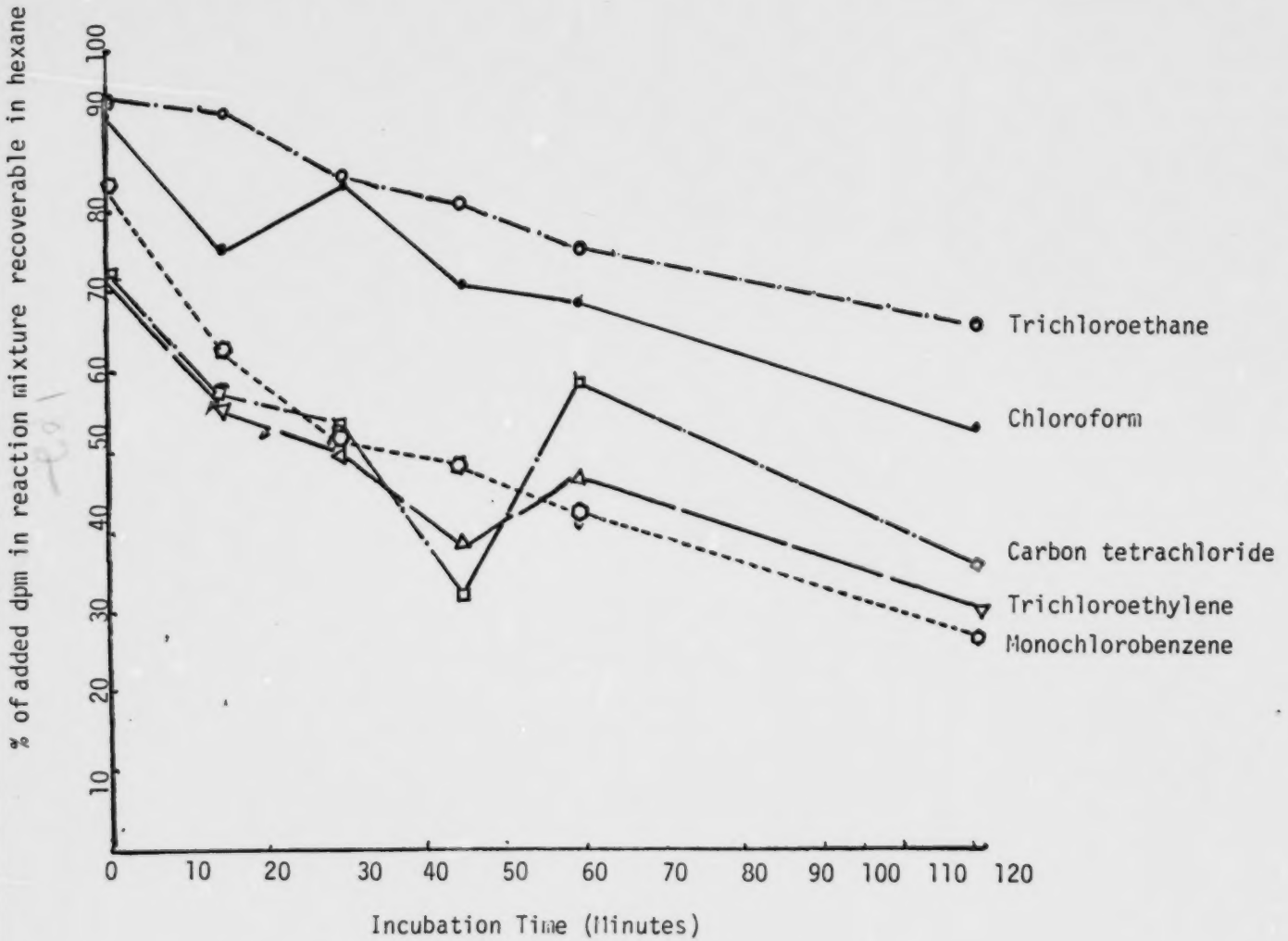


Figure 1. Disappearance of the added radioactivity from the reaction mixture at different time intervals.

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